USE OF AMINO ACID ANALOGUE-RESISTANT CELL LINES FOR SELECTION OF PLANT SOMATIC HYBRIDS

BY

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1979

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Professor Indra K. Vasil for acting as Chairman of my Supervisory Committee, for providing laboratory facilities, and for many helpful discussions during this study. Thanks are also extended to Dr. H.C. Aldrich, Dr. L.C. Hannah, Dr. J.M. Gregg and Dr. F.C. Davis, Jr., for serving on my committee.

I acknowledge Dr. A.S. Bleiweis for use of his amino acid analyser, and Mr. Steve Hurst for conducting the analysis.

A New Zealand National Research Advisory Council Fellowship is also gratefully acknowledged.

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June, 1979

Chairman: Indra K. Vasil
Major Department: Microbiology and Cell Science

The objective of this study was to test the hypothesis that it is possible to select double resistant somatic cell hybrids after fusing protoplasts of different amino acid analogue-resistant higher plant cell lines. Nicotiana sylvestris cell lines resistant to S-2-aminoethyl-cysteine (AEC^R), or 5-methyl-tryptophan ($5MT^R$), were isolated in suspension culture, from 6 x 10^6 cells, and 10^7 cells, respectively. These resistant cell lines required in excess of 100 ($5MT^R$), or 1,000 (AEC^R) times as much of their respective analogues as the parental wild-type culture for total growth inhibition. The AEC^R cell line also exhibited resistance to 10 mM of lysine plus threonine, which was totally inhibitory to growth of the $5MT^R$ cell line.

Protoplasts from the resistant cell lines were isolated and cultured in the medium of Nagy and Maliga (Z. Pflanzenphysiol. 78,453. 1976), containing 0.4 mg/l 2,4-D, 0.03 mg/l kinetin, and 0.4 M sucrose as osmoticum. Approximately 40-70% of the AEC^R protoplasts divided by 10 days after plating and subsequently formed calli. However, $5MT^R$ protoplasts rarely formed calli, and only 1-5% did so when mixed with AEC^R protoplasts in control or fusion experiments.

Eight calli (0.8%) were selected on double analogue medium after heterokaryotic fusion of AEC^R and $5MT^R$ protoplasts. A total of 1.8 x 10^4 control calli from mixed AEC^R and $5MT^R$ protoplasts, and AEC^R and $5MT^R$ homokaryotic fusions, were placed on the double analogue selection, but none survived. There was no evidence for cross-feeding, high frequency appearance of double resistance within the parental cell lines, nor increased resistance with homokaryotic fusion. Double resistant AEC^R + $5MT^R$ calli appeared only after fusion of parental protoplasts. These hybrid cell lines retained double resistance after growing for 6 months in the absence of the analogues.

Analysis of the hybrid calli showed the AEC-resistance to be dominant, and the 5MT-resistance semidominant. The parental cell lines had a distinct and nonoverlapping difference in chromosome numbers, and three of the four hybrids examined had chromosome numbers higher than either parent. There was an intermediate expression of an unselected AEC^R cell line trait, the ability to synthesize chlorophyll in the normally inhibitory presence of 2,4-D. None of the protoplast-derived calli examined were auxin-independent in their growth. Analysis of free amino acid levels in parental and hybrid protoplast-derived calli indicated that there was no accumulation of free lysine. The $5MT^R$ cells appear to overproduce tryptophan and the hybrid cells have levels intermediate between that of the two parents.

Evidence presented here suggests that dominant amino acid analogue-resistant cell lines can be used to select intraspecific somatic hybrids.

INTRODUCTION

One of the major problems in higher plant genetics and breeding is the existence of sexual incompatibility. This barrier restricts genetic analysis and crop improvement by limiting gene combinations to closely related compatible species. In some cases sexual incompatibility can be overcome and new sexual hybrids obtained by in vitro pollination, ovule culture, embryo culture or the use of mentor pollen. However, it is only with the recent advent of techniques for somatic hybridization by protoplast fusion, that hybridizations involving systematically unrelated partners became a possibility (Gamborg 1976; Galun et al. 1977; Vasil et al. 1978).

Effective techniques for protoplast fusion using polyethylene glycol (PEG) (Kao and Michayluk 1974) or high Ca^{++} and high pH (Keller and Melchers 1973) treatments have been developed, and there appears to be no restriction as to the types or species of protoplasts that can be fused to form heterokaryons. Even plant-mammalian cell heterokaryons can be formed using PEG (Duijts et al. 1976; Jones et al. 1976; Willis et al. 1977).

The remaining methodological component of somatic hybridization, that of selecting the hybrid cell(s) from the more numerous parental cells, lags in development, and there is a need for selection systems designed specifically for intergeneric somatic hybridizations. In mammalian somatic cell genetics a number of selection systems using either drug resistance or enzyme deficient mutants have been developed

(Harris 1970; Ephrussi 1972; Davidson 1974; Pontecorvo 1975). To date, somatic hybrid plants have been selected using naturally occurring differential growth in media or sensitivity to drugs (Carlson et al. 1972; Smith et al. 1976; Chupeau et al. 1978; Power et al. 1976), complementing chlorophyll-deficient mutants (Melchers and Labib 1974; Melchers 1977; Cocking et al. 1977; Scheider 1977, 1978a; Dudits et al. 1977; Maliga et al. 1978), complementary nitrate reductase deficient cell lines (Glimelius et al. 1978), and combined kanamycin resistance and restoration of shoot forming potential (Maliga et al. 1977). These somatic hybridizations have been between sexually compatible species or very closely related sexually incompatible species. In the case of tomato + potato hybridization (Melchers et al. 1978), no selection was used, and morphologically distinguishable presumptive hybrids had to be confirmed by fraction 1 protein analysis.

An alternative method of somatic cell hybrid selection is the manual isolation of heterokaryons and their culture, as developed by Kao (1977). Leaf and suspension protoplasts are fused and their morphological markers used to identify heterokaryons. This technique requires the culture of single hybrid cells, at present only possible in a few species, and there is no selective pressure to maintain hybridity in the presence of chromosome elimination, which occurs in some wide somatic cell hybrids (Kao 1977; Binding and Nehls 1978).

Theoretically a selection system using dominant resistance could be applied for the isolation of intergeneric hybrids. A number of cell lines resistant to the growth inhibitory effects of amino acid analogues have been isolated (Carlson 1973; Widholm 1972b,c 1976; Chaleff and Carlson 1975). The mechanism of resistance in many of these

cell lines appears to be a relaxed feedback inhibition of control enzymes by the free amino acid, which dilutes the analogue. The present study attempts to determine if amino acid analogue resistant cell lines can be used to select double resistant somatic cell hybrids. To test this hypothesis an intraspecific somatic hybridization of Nicotiana sylvestris was attempted using separate cell lines resistant to 5-methyl tryptophan (5MT) or S-2-aminoethyl cysteine (AEC).

CHAPTER ONE

LITERATURE REVIEW

Amino Acid Analogue Resistant Cell Lines

In higher plants, as in bacteria, the synthesis of many amino acids has been shown to be controlled by feedback inhibition. Generally the end product amino acid inhibits activity of the first enzyme exclusive to the amino acid pathway. In this way the concentration of the amino acid is regulated by controlling its own biosynthesis. A mutation providing resistance to feedback inhibition by the end product amino acid could lead to increased levels of this free amino acid.

The tryptophan biosynthetic pathway operative in higher plants (Wightman et al. 1961; Yoshida and Towers 1963; Delmer and Mills 1968; Singh and Widholm 1974), is apparently the same as that elucidated in microorganisms (Sprinson 1960; Edwards et al. 1964). In higher plants anthranilate synthetase is controlled by feedback inhibition by tryptophan, and not by repression of enzyme synthesis (Belser et al. 1971; Widholm 1971). Analogues of tryptophan, including 5-methyltryptophan (5MT), inhibit growth of carrot and tobacco cells in culture, apparently by false feedback inhibition of anthranilate synthetase (AS; Widholm 1972a), and this inhibition can be reversed by adding tryptophan to the culture medium.

Widholm (1972b,c) isolated cell lines of tobacco and carrot resistant to normally growth inhibitory concentrations of 5MT. In both cases the resistant lines were recovered in suspension culture by incubating for

a prolonged period (30-60 days). The spontaneous frequency of appearance of resistant cells was about one cell in 6×10^5 for tobacco, and one in 3.6×10^6 for carrot. In carrot this frequency of resistant cells could be increased 10 to 20 fold by treatment with the mutagens ethylmethane sulfonate or ultraviolet light (Widholm 1977a). In carrot the resistance was usually stable, with resistant lines maintaining original levels of resistance when grown for 100 cell mass doublings in a medium lacking 5MT. However, some lines did lose the resistance trait after long term culture. The resistance of cell lines in most cases was due to an altered AS enzyme, less sensitive to inhibition by 5MT and by tryptophan. This relaxed feedback control of AS resulted in increased levels of free tryptophan, 27-fold in carrot cells, and 33-fold in tobacco cells (Widholm 1972c). In normal tobacco cells the free tryptophan level is about 2.7% of the total cellular tryptophan (Widholm 1977a). Therefore, a 33-fold increase in the free level in resistant cells resulted in a total increase of 86%.

One of the major reasons for isolating amino acid analogue resistant cell lines which overproduce and accumulate free amino acids is the prospect of regenerating plants having increased levels of essential amino acids. Man and other monogastric animals cannot synthesize certain amino acids, and their diet must contain these for correct nutrition. Therefore, it is important to determine whether the overproduction-accumulation traits are also expressed in plants regenerated from such cell lines. Unfortunately, many of the overproducing cell lines isolated lack regenerative capacity (Maliga 1978), presumably due to long periods in culture. Recently, Widholm (1978) regenerated plants from a 5MT resistant cell line with an altered AS enzyme. Cultures initiated from

the leaves retained the resistance, altered synthetase and increased tryptophan, but leaves of the plant did not contain the altered enzyme activity. It may be that different isoenzyme forms of AS are expressed in cell cultures and in the regenerated plant. Carlson and Widholm (1978) have described the separation of two forms of AS from 5MT resistant and susceptible lines of potato (Solanum tuberosum). AS from resistant cells was less sensitive to feedback inhibition by tryptophan and 5MT than the sensitive line, and there was a 48-fold increase in free tryptophan in one of the resistant cell lines. Preparative polyacrylamide gel electrophoresis separated feedback sensitive and resistant forms of AS in extracts from both 5MT resistant and susceptible cells, with a predominance of the corresponding form in the respective cell type. It is not clear whether the alteration providing resistance is due to mutation, possibly regulatory, or an adaptation. The resistance trait was shown to be stable and to occur at a frequency of about one in 10^6 to 10^7 cells.

A carrot cell line resistant to 5MT has also been regenerated (Widholm 1974a). Resistance to growth inhibition was apparently due to decreased uptake of the tryptophan analogue, and all but one culture reinitiated from 32 plants retained resistance. In order to demonstrate stability of resistance in carrot, two single cell clones were isolated and grown for over 250 generations away from 5MT. These cloned lines retained resistance identical to that of the original line grown continuously in 5MT, and its altered enzyme and elevated free tryptophan levels (Widholm 1977a).

Tryptophan is considered to be the precursor of the natural auxin indole-3-acetic acid (IAA; Schneider and Wightman 1974), and will alleviate the dependence on auxin for growth in carrot (Gautheret 1955),

and tobacco (Beauchesne 1974) cultures. Of 10 carrot lines resistant to 5MT, which accumulated free tryptophan, due to an altered AS, 5 were auxin-autotrophic. However, of several auxin-autotrophic carrot and potato lines selected from normal lines, none were resistant to 5MT (Widholm 1977b).

Carrot and tobacco suspension culture lines resistant to DL-p-fluorophenylalanine (PFP) were selected by incubating cells in medium containing growth inhibitory levels of the phenylalanine analogue for 2 months (Widholm 1974b; Palmer and Widholm 1975). This analogue apparently inhibits growth due to incorporation into protein (Widholm 1977a). The frequency of appearance of resistant cells was less than one in 10^7, and all 14 single cell clones obtained from the resistant carrot line retained resistance which was evidently due to oversynthesis and accumulation of phenylalanine. The resistant tobacco line apparently oversynthesized phenylalanine, since the cells had an altered chorismate mutase, the control enzyme. However, phenylalanine did not accumulate, but was converted into phenolics, which did accumulate. Gathercole and Street (1978) isolated PFP-resistant lines from a diploid Acer pseudoplatanus L. suspension culture and studied characteristics of one of the lines: no increase in free phenylalanine, increased phenolic levels, decreased PFP and phenylalanine uptake, and increased phenylalanine ammonia lyase levels, were similar to those described in the tobacco PFP-resistant cell line.

In higher plants lysine, threonine, isoleucine and methionine are synthesized from aspartate, via the aspartate pathway (Miflin 1973). Enzyme studies, and results of growth inhibition experiments, using the end product amino acids, indicate that feedback inhibition controls

biosynthesis. Activity of aspartate kinase (AK), the first enzyme in the pathway, can be inhibited by lysine (Cheshire and Miflin 1975; Shewry and Miflin 1977; Aarnes 1977; Bright et al. 1978a), threonine (Aarnes and Rognes 1974), or the concerted action of lysine and threonine (Dunham and Bryan 1971; Wong and Dennis 1973a). When lysine-threonine inhibition of AK in cultured tissue is compared with that in whole roots a reverse relationship is demonstrated. Two forms of AK have been found in extracts of whole carrot roots (Sakano and Komamine 1978). The lysine-sensitive form of the enzyme constituted less than 20% of the AK activity, and the threonine-sensitive about 70%. But in cultured root slices incubated for three days, the proportions of the two enzyme forms reversed. Similar results were obtained by Matthews and Widholm (1978) when comparing AK inhibition in established carrot suspension cultures and in whole roots.

Exogenously supplied lysine and threonine have been shown to inhibit growth in a synergistic manner, and this inhibition can be reversed by methionine or its metabolic precursors homoserine and homocysteine (Dunham and Bryan 1969; Henke et al. 1974; Bright et al. 1978a; Green and Phillips 1974). It has been suggested that this effect of lysine plus threonine could be due to their cooperative inhibition of AK (Wong and Dennis 1973b), which in turn shuts off methionine biosynthesis. However, in barley, maize and wheat, the isolated AK enzyme is inhibited only by lysine (Cheshire and Miflin 1975; Shewry and Miflin 1977; Bright et al. 1978b). The branch point enzyme in the pathway for lysine biosynthesis is dihydrodipicolinic acid synthetase (Cheshire and Miflin 1975; Mazelis et al. 1977; Matthews and Widholm 1978), and this enzyme is inhibited by lysine only.

In an attempt to obtain lysine overproducing cell lines and plants a number of selections for lysine analogue resistants have been made. Using the lysine analogue aminoethylcysteine (AEC), Chaleff and Carlson (1975) isolated three resistant cell lines of rice (Oryza sativa L.) from cells mutagenized with ethylmethane sulfonate. These lines grew very slowly in concentrations of the analogue lethal to wild-type cells, and accumulated about twice the normal level of free lysine. There were also increases in isoleucine, methionine, and the nonaspartate derived leucine, valine, tyrosine and alanine. Unexpectedly, the resistant lines also had increases in the relative amount of lysine, isoleucine, leucine and valine incorporated into protein. Carrot cell lines resistant to AEC, or aminocapylic acid, another lysine analogue, have also been isolated (Miflin 1975). These lines do not accumulate free lysine, but contain aminoadipic acid and pipecolic acid which are products of lysine degradation. Widholm (1976) also used AEC or delta-hydroxylysine (DHL), another lysine analogue, to isolate resistant cell lines of tobacco after mutagenic treatment with ethylmethane sulfonate. Both the AEC and DHL resistant lines were cross resistant to AEC and DHL. The AEC resistant line was inhibited by lysine plus threonine, as is the normal line, but the DHL resistant cell line is not inhibited. Both the AEC and DHL resistant cell lines showed about a 10-fold increase in free lysine. AEC resistant cell lines of Arabidopsis thaliana do not show overproduction of lysine, or reduced uptake of AEC, but have a reduced incorporation of the analogue into proteins (Negrutiu et al. 1978).

In order to examine amino acid analogue resistance in whole plants, attempts have been made to use totipotent systems, embryo culture selection, or selection at the whole plant level. Barley mutants

resistant to AEC were selected from M2 embryos (Bright and Miflin 1978), and in one recessive mutant resistance was maintained for at least two generations. Uptake of ^{3}H-lysine was reduced, and there was no increase in lysine production in the whole plant.

Carlson (1973) selected tobacco cells resistant to methionine sulfoximine (MS), and regenerated plants from these cells. Some of the plants contained a 5-fold increase in free methionine, and the resistance was inherited in a semidominant manner. While the inhibitory effect of MS can not be reversed by exogenous methionine (Singh and Widholm 1974; Dessauer and Hannah 1978), indicating that it may not be a methionine analogue, these resistant plants do accumulate increased free methionine. Using an approach other than the usual one-step selection Zenk (1974) gradually adapted haploid-derived Nicotiana sylvestris suspension cultures to increasing levels of DL-ethionine (methionine analogue). This analogue was also used by Dessauser and Hannah (1978) to screen M2 seedlings from mutagen treated seeds of Vigna unguiculata for resistance. None of the resistant progeny from a single resistant plant contained increased levels of free or total methionine in the roots, shoots or seeds. A carrot suspension cell line resistant to ethionine and showing a 10-fold increase in free methionine has also been isolated (Widholm 1976).

Using sequential selection for resistance it has been possible to isolate a cell line simultaneously resistant to PFP, ethionine, AEC, and 5MT (Widholm 1978). A cloned line resistant to PFP previously described (Widholm 1977a) was used as the initial material and different resistance frequencies observed at each step in the selection. As resistance to each analogue was gained, the level of the corresponding

free amino acid also increased. However, after selection for ethionine resistance there was also a 2- to 3-fold increase in lysine, aspartate and tryptophan, and some gain in resistance to AEC and 5MT. But it was not until 5MT resistance was selected that the AS enzyme with altered feedback inhibition properties appeared. Apparently, selection of amino acid analogue resistance can produce increases in free amino acids other than the "target" amino acid.

Recently, Bourgin (1978) selected valine resistant calli from ultraviolet treated haploid mesophyll protoplasts of tobacco. Valine at 0.1 to 10 mM is normally growth inhibitory (Bourgin 1976), and this inhibition can be relieved by simultaneous addition of isoleucine, suggesting interference in isoleucine bisynthesis. Among recovered plants two were resistant and transmitted the trait in sexual crosses as a mendelian character. In each case the resistance could be explained by a single allele, dominant in one and semidominant in the other.

Protoplast Fusion

One of the major steps in plant somatic hybridization is the fusion of protoplasts. There are two distinctly different types of protoplast fusion: spontaneous fusion and induced fusion (Power et al. 1970). The fusion of plant protoplasts during enzymatic degradation of cell walls has been attributed to the expansion of plasmodesmata between protoplasts derived from adjacent cells (Cocking 1972). Fusion between protoplasts from two different sources must be induced and requires molecular contact between membranes (Poste and Allison 1973).

Attempts to fuse mechanically isolated protoplasts were made early in the century by Winkler, Kuster, and Michel (in Power et al. 1970;

Melchers and Labib 1974). Subsequently, attempts were made to obtain reproducible, high frequency, controlled fusions of enzymatically isolated protoplasts. These included the treatment of protoplasts with sodium nitrate (Power et al. 1970), artificial seawater (Eriksson 1971), and deplasmolyzing osmotic shock (Keller et al. 1973). These procedures produced only low fusion frequencies and were generally not conducive to continued protoplast viability (Potrykus 1972; Carlson et al. 1972). In the case of protoplasts from meiotic cells a high incidence of spontaneous fusion occurs simply on contact, without the aid of any inducing agent (Ito 1973).

High frequency of protoplast fusion was first obtained by Keller and Melchers (1973) using a high pH (up to 10.5) and high calcium ion solution at 37°C. High molecular weight polyethylene glycol (PEG; 1,000-6,000 MW), introduced by Wallin et al. (1974), and Kao and Michayluk (1974), also induces close contact and high frequency nonspecific fusion, and has been effective for fusion of protoplasts from a large number of species, from different genera and families (Kao et al. 1974; Kartha et al. 1974; Constabel et al. 1975). Heterokaryon formation frequencies of up to 50% have been achieved by eluting the PEG with a solution of high pH, and high calcium ion concentration (Burgess and Fleming 1974; Kao et al. 1974; Wallin et al. 1974; Kao 1977).

While the mechanism of PEG mediated fusion is not well understood a mode of action has been proposed (Constabel and Kao 1974; Grout and Coutts 1974; Kao and Michayluk 1974; Wallin et al. 1974; Kao and Wetter 1977). The general formula of PEG is $HOCH_2-(CH_2-O-CH_2)_n-CH_2OH$, and even high molecular weight molecules are very water soluble. The molecule is negative in polarity and capable of forming hydrogen bonds

with water, carbohydrates, proteins, etc., which have positively charged groups. It is proposed that when the PEG molecules are long (1,000 MW) they act as a molecular bridge between adjacent membranes and adhesion occurs. PEG can bind Ca^{++} which may form a link between negatively polarized groups of phospholipid or protein, and the PEG. During washing the PEG molecules binding the protoplast membranes together could be eluted and result in a redistribution of charges between the membranes which are in close contact over large surface areas (Burgess and Fleming 1974; Fowke et al. 1975).

More recently attempts have been made to enhance PEG-induced fusion, increase heterokaryon viability and reduce loss of fusion products during washing. Haydu et al. (1977) found that treatment with dimethyl sulfoxide (DMSO) significantly increased the frequency of fusion induced by PEG and had no inhibitory effect on the division of cells. The viability of protoplasts after PEG treatment can be enhanced by determining the optimum pH for the PEG treatment (Gosch and Reinert 1978). By using Petri dishes coated with concanavalin A the attachment of protoplasts induced by PEG can be increased in tobacco (Glimelius et al. 1978), and due to this stronger attachment more fusion products were retained after the dilution and washing procedures.

As the above short review indicates, efficient, high frequency methods of protoplast fusion are available, and there is no barrier to the fusion of any two types of protoplasts and the formation of heterokaryons. This is well demonstrated by the viability of such wide heterokaryons as Zea mays + Glycine max (Kao et al. 1974), and plant-animal heterokaryons such as Daucus carota + human cells (Dudits et al. 1976).

Selection of Somatic Hybrids

Fusion of protoplasts and heterokaryon formation are only the first, and probably the easiest, steps in the formation of a proliferating hybrid cell line. A number of factors may operate which lead to the loss of many of the heterokaryons, such as overgrowth by more rapidly growing parental cells, and asynchronous division leading to chromosome elimination. Therefore, in order to recover rare hybrid colonies from a very large population of parental types, it is necessary to have a stringent selection system. Although the first somatic hybrids were reported very recently (Carlson et al. 1972), there has been rapid progress in this field of research (Cocking 1972, 1976, 1978; Gamborg et al. 1974a;Gamborg et al. 1974b;Vasil 1976; Gamborg 1976, 1977; Bajaj 1977; Bhojwani et al. 1977; Galun et al. 1977; Melchers 1977; Constabel 1978; Vasil et al. 1978).

To date, only a limited number of selection systems have been used for the isolation of somatic cell hybrids and hybrid plants. Those cases which have resulted in the isolation of somatic hybrid plants are listed in Tables 1 and 2. One approach has been to use naturally occurring differences between the growth of hybrid and parental cells or protoplasts on a selective medium. Using growth differences between mesophyll protoplasts of the parents and those of the sexual hybrid, Carlson et al. (1972) selected somatic hybrid plants of *Nicotiana glauca* + *N. langsdorffii*. Protoplasts of the two parental species do not regenerate into callus in the medium used, while callus develops from about 0.01% of the sexual hybrid protoplasts. The ability of hybrid calli to grow on medium without added phytohormones was used as a second selective step. In regenerated plants morphological characteristics, chromosome numbers, and peroxidase

Table 1. Methods of selecting somatic hybrid plants. I. Use of chlorophyll-deficient mutants.

Somatic hybrid	Selection method	Reference
Datura innoxia + *D. innoxia*	Albino complementation to green	Schieder, 1977
Datura innoxia + *D. discolor**	Albino regenerating + green nonregenerating, complementation to green regenerating	Schieder, 1978a
Datura innoxia + *D. stramonium**	Albino regenerating + green nonregenerating, complementation to green regenerating	Schieder, 1978a
Daucus carota + *D. capillifolius*	Albino regenerating + green nonregenerating, complementation to green regenerating	Dudits et al., 1977
Daucus carota + *Aegopodium podograria**	Albino regenerating + green nonregenerating, complementation to green regenerating	Dudits et al., 1978
Nicotiana tabacum + *N. knightiana*	Albino regenerating + green nonregenerating, complementation to green regenerating	Maliga et al., 1978
Nicotiana tabacum + *N. sylvestris*	Light sensitive, chlorophyll deficient, complementation to green, light resistant	Melchers, 1977
Nicotiana tabacum + *N. tabacum*	Light sensitive, chlorophyll deficient, complementation to green, light resistant	Melchers and Labib, 1974 Gleba et al., 1975
Petunia hybrida + *P. parodii*	Albino regenerating + green nonregenerating, complementation to green regenerating	Cocking et al., 1977
Solanum tuberosum + *Lycopersicon esculentum**	No invitro selection, albino tomato, selection of regenerants with intermediate characteristics	Melchers et al., 1978

*sexually incompatible combinations

Table 2. Methods of selecting somatic hybrid plants. II. Use of selective media and biochemical mutants.

Somatic hybrid	Selection method	Reference
Nicotiana glauca + *N. langsdorffii*	Parents auxin-dependent for growth, hybrids auxin-autotrophic, selection on medium without phytohormones	Carlson et al., 1972
Nicotiana sylvestris + *N. knightiana*	Kanamycin resistance + nonregeneration, selection for resistance and regeneration	Maliga et al., 1977
Nicotiana tabacum + *N. tabacum*	Nitrate reductase deficient mutants, complementation to wild-type	Glimelius et al., 1978
Petunia hybrida + *P. parodii*	Regenerating parent sensitive to actinomycin D + nonregenerating parent resistant to actinomycin D, selection for resistance and regeneration	Power et al., 1976

isozymes of the somatic hybrids were identical to those of the sexual amphidiploid. Analysis of fraction 1 protein from one somatic hybrid showed the presence of the small subunit polypeptides (nuclear coded) of both parents, but only the subunit polypeptide (cytoplasmically coded) of N. glauca (Kung et al. 1975). Smith et al. (1976) extended this model somatic hybridization system, using an improved method of fusion (PEG), and an enriched protoplast culture medium which allowed slow growth of parental protoplasts but rapid growth of hybrid cells. In this case selection was solely on medium lacking phytohormones. Of 23 mature hybrid plants obtained, chromosome numbers ranged from 56 to 64, suggesting triple fusion (giving 60-66 chromosomes) followed by aneuploidy introduced during callus growth, and possibly selection for a range of chromosome numbers favorable to regeneration. Analysis of fraction 1 protein in these hybrids showed that all had small subunits of the parents, but about half of the plants had large subunits of N. glauca, and the others of N. langsdorffii. One weakly growing plant contained large subunits of both parents. Using the same system, Chupeau et al. (1978) regenerated 6 somatic hybrid plants. Two had 42 chromosomes and were morphologically identical to the sexual amphidiploid.

Power et al. (1976) selected somatic hybrids of Petunia hybrida + P. parodii using natural resistance/sensitivity to actinomycin D and a difference in the ability of protoplasts for sustained growth. Petunia parodii mesophyll protoplasts can divide and grow in the presence of 1.0 ug/ml of actinomycin D, but do not grow beyond the small colony stage. Protoplasts of P. hybrida are killed by this concentration of the drug, but in its absence will sustain division and can be regenerated. Hybrids were selected due to their ability to sustain division in the presence

of actinomycin D. Plants regenerated from selected calli were classified as hybrids on the basis of flower color and shape, and peroxidase isozymes. This complementation/selection procedure was not based upon a knowledge of growth requirements for protoplasts of the sexual hybrid.

Complementing chlorophyll-deficient, light sensitive, haploid mutants were used by Melchers and Labib (1974) to select green, intraspecific somatic hybrids of Nicotiana tabacum resistant to high light intensity. The two recessive mutants, sublethal (s) and virescent (v), had previously been shown to complement to wild-type in sexual hybrids. The somatic hybrids obtained were further identified by segregation of the recessive mutants in the F2 progeny, thus demonstrating that the normal green, light-resistant plants could not be revertants of v or s. A similar selection system has been used to obtain somatic hybrid plants of N. tabacum + N. sylvestris (Melchers 1977). Here the complementation of the two chlorophyll-deficient mutants was detected only by somatic hybridization. Gleba et al. (1975) also selected N. tabacum intraspecific somatic hybrids, but the complementation was between plastome and genome chlorophyll mutants which were not light sensitive. Following fusion between protoplasts from two nonallelic diploid albino mutants of Datura innoxia it was possible to select a large number of green calli (Schieder 1977). In extensive control experiments involving mixed protoplasts of the two mutants no green calli were obtained, and no back mutation occurred in long term shoot cultures. Of 20 regenerated plants, 5 were tetraploid, 8 hexaploid, 3 octoploid, and 4 showed an aneuploid chromosome number. Genetic evidence for the hybrid nature of these somatic hybrids was demonstrated with the aid of anther culture, where both green and chlorophyll-deficient androgenetic lines were regenerated from pollen

grains (Schieder 1978b). The same albino complementation to green has been used to select interspecific Petunia hybrida + P. parodii hybrids (Cocking 1978).

To date, many of the somatic hybrids obtained have been selected using modifications of the albino complementation procedure. Cocking et al. (1977) selected interspecific somatic hybrids of Petunia hybrida + P. parodii using albino, regenerating P. hybrida protoplasts and wild-type P. parodii protoplasts unable to sustain division after forming small colonies. Selection was for somatic hybrids with normal chlorophyll synthesis, and the ability to sustain division and regenerate. Similarly, Dudits et al. (1977) isolated somatic hybrids of Daucus carota + D. capillifolius. But in this case a few green D. capillifolius plants could develop from protoplast cultures. Therefore, it was necessary to use morphological markers, and peroxidase isozymes, to identify the somatic hybrids. The hybrids were intermediate between the parents in overall appearance, but some had chromosome numbers which varied slightly from the expected. Somatic hybrids of Nicotiana knightiana and an albino mutant of N. tabacum were selected on the basis of simultaneous expression of shoot inducibility and green pigmentation, traits originally separated in the two parental species (Maliga et al. 1978). The dominance of shoot inducibility had previously been established in sexual hybrids. There is a difference in appearance of heterochromatin blocks in the interphase nuclei of the parents (Nagy et al. 1977), and the hybrid lines were confirmed using this character, isozyme patterns, and the morphology of regenerated plants. Chromosome numbers in the somatic plants varied greatly within individual plants, and variegation in leaf and flower color, and segregation for morphological traits in vegetatively multiplied

plants, was attributed to segregation of chromosomes in somatic cells, due to the numerical instability.

In all the above cases the somatic hybrids obtained were between sexually compatible parents. This type of hybridization may be useful for studying cytoplasmic inheritance and the expression of mutants which cannot be regenerated, but it is the somatic hybridization of sexually incompatible parents which is of agronomic importance. Recently four such combinations have been obtained. After fusion of protoplasts from a chlorophyll-deficient diploid mutant of Datura innoxia which can be regenerated, with green wild-type protoplasts of Datura stramonium which can not, Schieder (1978a) isolated green calli and regenerated plants. Chromosome counts of the somatic hybrids showed that 15 were tetraploid (amphidiploid), 24 hexaploid, and one aneuploid. The same selective procedure was used to obtain four D. innoxia + D. discolor somatic hybrid plants, 3 of which were amphidiploid and one aneuploid. The habits of the hybrids in both combinations were intermediate between those of the parental species. All hybrids were fully fertile, set seed, and exhibited hybrid vigor at both the callus and plant level. The fact that such hybrids can be obtained indicates that it is not necessary for protoplasts of both species to be able to regenerate. While normal hybridization in both combinations does not occur, a few diploid hybrids have been obtained by embryo culture (Rietsema and Satina 1959).

In a recent report Melchers et al. (1978) describe the isolation of four potato + tomato somatic hybrid plants. Albino leaf protoplasts were used to screen out the tomato parent, but no selection system against the potato parent was used. Possible hybrids were selected on the basis of a difference from the parents in leaf shape, and flower

morphology and color. The hybrid nature of these plants was demonstrated by fraction 1 protein analysis. All four plants contained the small subunits (nuclear) of both potato and tomato. In three plants the large subunit (cytoplasmic) was that of tomato, whereas in the fourth it was derived from potato. The latter hybrid had a chromosome number near 72 (possible triple fusion), while the others were close to the expected 48 of an amphidiploid. At present this hybrid can not be obtained sexually.

An intergeneric hybridization between Daucus carota and Aegopodium podograria has been reported by Dudits et al. (1978). Regenerating albino carrot suspension and nonregenerating A. podograria leaf protoplasts were fused and plants regenerated from three selected green calli. Although the selected plants possessed only carrot chromosomes, the restoration of chloroplast ultrastructure, synthesis of photosynthetic pigments, and expression of A. podograria markers for root development and root carotenoids, indicated the presence of A. podograria genes. This could be due to the elimination of A. podograria chromosomes and the insertion of a few A. podograria genes into the carrot genome. It should be noted that a number of attempts using chlorophyll-deficient mutants have as yet been unsuccessful. These include Petunia hybrida + Nicotiana tabacum (Zenkteler and Melchers 1978), Datura innoxia + Nicotiana sylvestris and D. innoxia + P. hybrida (Schieder 1977), and some as yet unpublished attempted wide hybridizations (Cocking 1978).

In order to study chromosomal behavior in somatic hybrids between wide combinations, Kao (1977) developed a technique for mechanical isolation and culture of single heterokaryons. Suspension protoplasts of soybean were fused with mesophyll protoplasts of Nicotiana glauca

and the morphologically distinct heterokaryons isolated and cultured in a complex medium. In the first few cell generations the N. glauca chromosomes had a tendency to break or stick together. However, only a few of the N. glauca chromosomes were retained in the somatic hybrids after 6 months in culture. Examination of chromosomes and isozyme patterns in a number of hybrid isolates indicated that the elimination of N. glauca chromosomes was random (Kao 1977; Wetter 1977). Similarly, Binding and Nehls (1978) observed elimination of most of the chromosomes of one or the other species in hybrid calli of Vicia faba + Petunia hybrida. In contrast to these results, the mechanically isolated somatic cell hybrids of Arabidopsis thaliana + Brassica campestris retained chromosomes of both the parents after 7 months in culture (Gleba and Hoffman 1978). Reconstituted di- and multiconstructional chromosomes were observed, similar to those of Kao (1977), but biochemical analysis revealed isozymes of both parents to be present in all hybrid cell lines. Therefore, in this case considerable stability, with retention and activity of chromosomes of both the species for at least 7 months, has been demonstrated. Unfortunately, the major prerequisite for selection by mechanical isolation--growth of single protoplasts in small volumes of medium--is at present restricted to only a few species.

In mammalian somatic cell genetics, enzyme deficient and drug resistant mutants have been used to isolate somatic cell hybrids. To date, only a limited use has been made of such approaches in higher plant somatic hybridization. In order to genetically analyze a kanamycin resistant Nicotiana sylvestris cell line deficient in inducible shoot redifferentiation, Maliga et al. (1977) selected N. knightiana + N. sylvestris somatic hybrids by screening for kanamycin resistance and green

pigmentation. In the hybrids shoot formation on inductive medium was restored. Glimelius et al. (1978) were able to select wild-type Nicotiana tabacum somatic hybrids after fusing two complementing, chlorate resistant mutant cell lines lacking nitrate reductase, and auxotrophic for reduced nitrogen. Hybrids were detected by their regained ability to grow on medium containing nitrate as the sole nitrogen source. Back mutation and crossfeeding were excluded as possible explanations for the occurrence of cell lines utilizing nitrate. The hybrids regained shoot inducibility and chlorate sensitivity.

Selection for a hybrid cytoplasm with only one of the nuclear genomes present (cybrid) is also possible. Plastome chlorophyll deficiency markers and nuclear genome markers have been used to obtain cybrids of N. tabacum (Gleba et al. 1975), and N. tabacum + N. debneyi (Gleba 1978). Cybrids of two N. tabacum varieties differing in their cytoplasms have been used to study chloroplast DNA (cpDNA) distribution and the mechanism of cytoplasmic male sterility (Belliard et al. 1978). Protoplasts of the variety Techne, characterized by a nuclear N. tabacum genome and N. debneyi cytoplasm and cytoplasmic male sterility, were fused with protoplasts of N. tabacum var. Samsun, and the cybrids identified using morphological markers. Analysis of cpDNA using Eco RI restriction nuclease revealed that only one or other of the parental cpDNAs is present in all cases. Results indicated that cpDNA is independent from the mechanism of cytoplasmic male sterility in tobacco. Cybrids of N. sylvestris and N. tabacum have been selected after irradiating the N. tabacum protoplasts to eliminate its nuclear genome (Zelcer et al. 1978). Male sterility was transferred from N. tabacum to the cybrid.

CHAPTER TWO

ISOLATION AND PARTIAL CHARACTERIZATION OF AMINO ACID ANALOGUE RESISTANT CELL LINES

Introduction

The objective of these experiments was to isolate separate S-2-aminoethyl-cysteine and 5-methyl-tryptophan resistant cell lines of Nicotiana sylvestris suitable for testing the hypothesis that amino acid analogue resistant cell lines can be used to select double resistant somatic cell hybrids. For such a use the following criteria were examined: (a) That the AEC resistant cell line be able to grow without inhibition in a concentration of AEC lethal to the 5MT resistant cell line, and vice versa. (b) That the selected cell lines retain stable resistance both in the presence and absence of the analogues. In order to gain some insight in to mechanism of AEC resistance, a study of the growth inhibitory effects of end product amino acids was made, and the levels of free amino acids in the wild-type and resistant cell lines were determined.

Materials and Methods

Petioles of diploid Nicotiana sylvestris Speg & Comes. were used for callus initiation. The excised petioles were sterilized by immersion in 70% ethanol for 3 mins followed by 7% calcium hypochlorite for another 3 mins. All further manipulations were carried out under sterile conditions in a laminar flow cabinet. After 3 washes in sterile distilled water,

3-5 mm transverse segments were placed on callus induction medium solidified with 0.8% Difco agar, 50 ml per 150 ml Erlenmeyer flask. All media and materials were sterilized by autoclaving at 15 psi for 15 mins. Both callus and suspension cultures were grown in Linsmaier and Skoog's (1965) medium containing 0.4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.03 mg/l kinetin, which promotes very friable and rapid growth. Suspension cultures were initiated with 2-3 g of callus in 40 ml of liquid medium per 250 ml Erlenmeyer flask. The suspension cultures of cell lines used in this study were maintained in 250 ml Erlenmeyer flasks on a gyrotory shaker (120 rpm). Ten ml of cell suspension was diluted with 40 ml of fresh medium at four day intervals. Both callus and suspension cultures were incubated at 25°C in the dark.

Growth studies on suspension cultures were made using 0.8 g fresh weight inoculum in 40 ml of fresh medium, grown for 10 days. Inhibitors (analogues or amino acids) were incorporated into the medium prior to autoclaving. Three or four replicates were made of each treatment. Fresh weight increases were determined after collecting cells and cell clumps on a Millipore absorbant filter pad by vacuum filtration. Cell counts were made using the chromium trioxide maceration technique of Henshaw et al. (1966), and a corpuscle counting chamber. After dilution a total of 16, 10 µl samples were counted and the mean used to calculate the original cell number/ml.

Growth of the wild-type N. sylvestris suspensions was totally inhibited by either 10 µg/ml of AEC (Calbiochem) or DL-5MT (Sigma) when the growth period was 10 days. Spontaneous resistant variants were isolated by incubating for 6-8 weeks in 10 µg/ml of analogue, at which time a few small cell clumps appeared and proliferated. The cell lines selected for use (AEC^R

or $5MT^R$) grew at normal or more rapid rates when reinoculated into 10 µg/ml of their respective analogues, and were thereafter maintained by diluting 10 ml into 40 ml of fresh medium containing 50 µg/ml of the analogue every four days. The AEC^R cell line described here was isolated in January 1976, at Grasslands Division, DSIR, New Zealand, and plated by the soft agar over-lay technique to form callus, for the transfer to Florida. Suspensions were diluted 1:4 in fresh medium containing 10 µg/ml AEC and 0.6% agar, and 5 ml overlayed on 20 ml of solidified (1.0% agar) medium in 100 x 15 mm plastic petri dishes.

Growth of the AEC^R cell line is totally inhibited by 1300 ug/ml of AEC. A cell line able to grow in the presence of 1500 ug/ml of AEC was isolated from the AEC^R line, subdivided into two separate cultures, one grown in the presence of 50 ug/ml of AEC, and the other without analogue. These were maintained for over 6 months and then tested for their levels of AEC-resistance.

Attempts to regenerate shoots from the wild-type and resistant cell lines were made using the RMO and RMB media of Maliga et al. (1977).

Free amino acids were extracted from exponentially growing cells which had been cultured away from the analogue for at least 3 subcultures (about 12 generations). Cells were collected by vacuum filtration and the extraction made using methanol-chloroform-water (12:5:1 v/v) as described by Singh et al. (1973). Twenty grams fresh weight of tissue was homogenized in a Pyrex 40 ml glass tissue grinder with 20 ml of methanol-chloroform-water, and after centrifugation at 10,000 x g for 5 mins, the pellet was reextracted with the same volume. A further 15 ml of water plus 10 ml of chloroform was added to the pooled supernatants and the aqueous phase taken to dryness under vacuum (15 cm of mercury) at 45°C.

The residue was dissolved in 5 ml of 0.01 N HCl and the samples analyzed on a two column automated Jeolco JLC-6AH amino acid analyzer. On each run of the samples a Pierce amino acid standard diluted to 100 nm/ml was used for calibration. Areas under the peaks were obtained using a Spectrophysics autolab computing integrator. All measurements were duplicated.

Chromosome numbers were counted in squash preparations of rapidly growing cell suspensions stained with 45% acetocarmine. In a few cases cells were pretreated in 0.05% colchicine for 15 mins at room temperature followed by 45 mins at 14°C, in order to obtain a better spreading of the chromosomes and to study their morphology.

Results

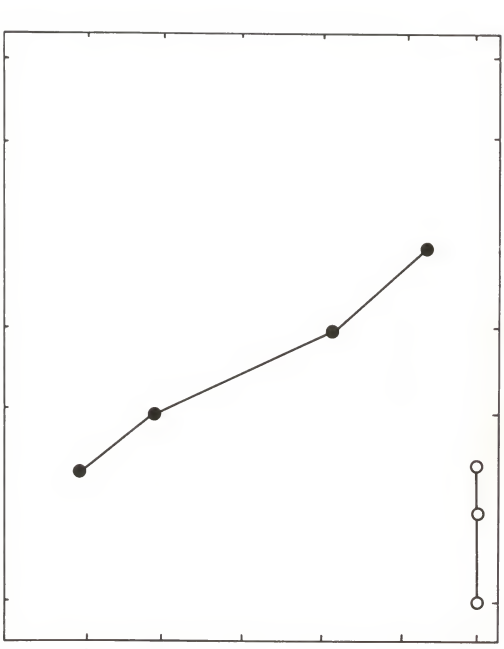
Amino acid analogue resistant cell lines: Preliminary experiments demonstrated that the growth of normal Nicotiana sylvestris suspension cultures was inhibited by 10 µg/ml of AEC. A total population of 6×10^6 cells was placed under this selection and after 6 weeks a few small cell clumps proliferated in one of three flasks. When tested for resistance, this cell line (AEC^R) showed no growth inhibition in 200 µg/ml of AEC, and about a 50% reduction in growth in 1,000 µg/ml AEC (Fig. 1). The AEC^R cell line requires in excess of 1,000 times as much analogue as the parent culture for total growth inhibition. A normal level of sensitivity to 5MT was retained (Fig. 2).

A $5MT^R$ cell line was selected in 10 µg/ml of 5MT from about 10^7 cells, appearing 4 weeks after inoculation. Cells proliferated rapidly in one of four flasks and this line was tested for 5MT resistance (Fig. 2). Growth of the $5MT^R$ cell line was not inhibited by 30 µg/ml of 5MT, but

Fig. 1. Effect of S-2-aminoethyl-cysteine on the growth of AEC^R (o) and $5MT^R$ (●) cells. Values are the increase in fresh weight given as a percentage of the same line grown in the absence of the analogue. Period of growth 10 days.

FRESH WEIGHT INCREASE (% OF CONTROL)
0
20
40
60
80
100
10
50
100
500
1000
S-AMINOETHYL-L-CYSTEINE (μg/ml)

Fig. 2. Effect of 5-methyl-tryptophan on the growth of $5MT^R$ (●) and AEC^R (o) cells. Values are the increase in fresh weight given as a percentage of the same line grown in the absence of the analogue. Period of growth 10 days.


FRESH WEIGHT INCREASE (% OF CONTROL)
0
20
40
60
80
100
10
50
100
500
1000
5-METHYL TRYPTOPHAN (µg/ml)

was 60% inhibited by 100 µg/ml. This line also exhibited a slightly higher level of resistance to AEC than the parent culture, with 80% growth inhibition in 10 µg/ml and total inhibition in 75 µg/ml of AEC (Fig. 1).

The AEC^R and $5MT^R$ cell lines show distinct differences in pigmentation and cell aggregate morphology. Suspension cultures of the AEC^R cell line grown predominantly in the dark, with short periods of light daily, are bright yellow in color, in contrast to the dull white of the wild-type and $5MT^R$ cell lines. This character has been uniform and stable for three years, in the presence or absence of AEC. The AEC^R pigmentation changes to bright green when suspension or protoplast-derived callus cultures are grown in the presence of about 2,000 lux of continuous light. The analysis of chlorophyll levels in protoplast-derived calli of the AEC^R and $5MT^R$ cell lines is given in Chapter Two. In the presence of light the wild-type and $5MT^R$ cultures remain dull white. The $5MT^R$ cell line exhibited the same cell aggregate morphology as the parent suspension culture, with a proportion of the cells growing in chains, and the cells being irregular in shape (Fig. 4). In contrast to this the AEC^R cell aggregates are more compact, and comprised mainly of round thin-walled cells (Fig. 5). This characteristic was also stable for three years, in both the presence or absence of the analogue.

An AEC-resistant line (designated AEC^R1500) capable of growth in 1,500 ug/ml of AEC was isolated from cells of the AEC^R line, appearing 6 weeks after inoculation. This line showed a slight decrease in AEC-resistance level after growing in the absence of any analogue for 6 months (Fig. 3).

Fig. 3. Effect of S-2-aminoethyl-cysteine on the growth of AEC^R (o), and AEC^R1500 cells grown for 6 months in the presence (▲), or absence (▲), of 50 ug/ml of AEC. Values are the increase in fresh weight given as a percentage of the same line grown in the absence of the analogue. Period of growth 10 days.

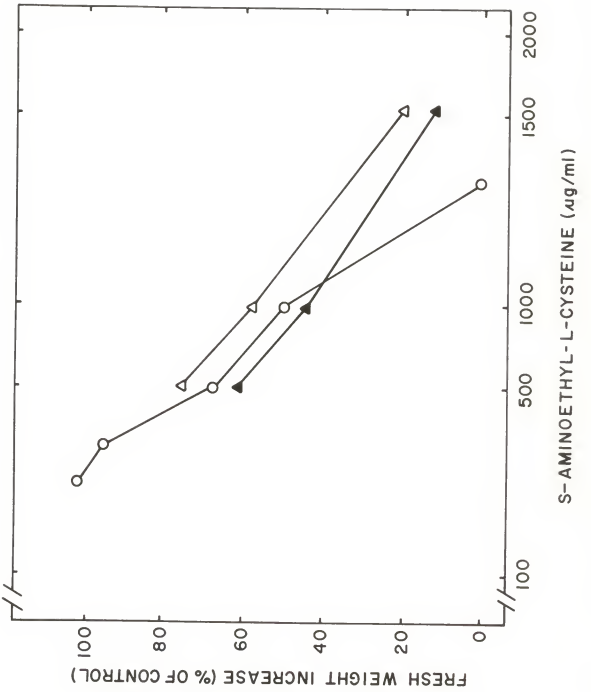
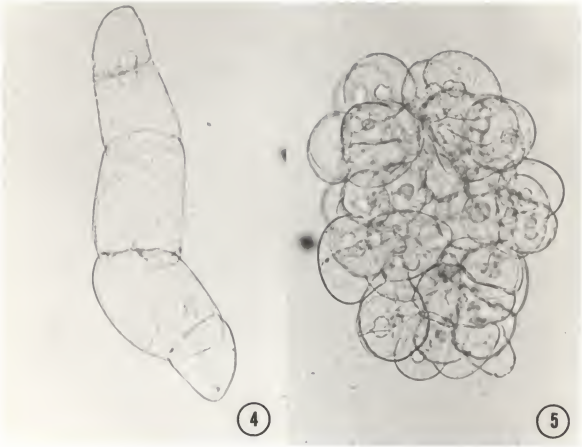

FRESH WEIGHT INCREASE (% OF CONTROL)
100
80
60
40
20
0
100
500
1000
1500
2000
S-AMINOETHYL-L-CYSTEINE (ug/ml)

Fig. 4. $5MT^R$ suspension cell aggregate. x 750

Fig. 5. AEC^R suspension cell aggregate. x 650


4
5

Attempts to regenerate shoots from the two-year old AEC^R and $5MT^R$ cell lines were unsuccessful. A few calli formed tight green regions after prolonged periods in the shoot induction medium but these only formed loose callus when transferred to fresh medium.

Growth inhibition by aspartate pathway end-product amino acids: Table 3 shows the growth inhibition, or lack of growth inhibition, by 1.0 mM of the aspartate derived amino acids, lysine, threonine, methionine, and combinations of these, of the wild-type, AEC^R and $5MT^R$ cell lines. At this concentration lysine had no marked growth inhibitory effect on any of the cell lines. Threonine partially inhibited growth of the wild-type and $5MT^R$ cell lines, but was not inhibitory to growth of the AEC^R line. Methionine at 1.0 mM partially inhibited growth of all three cell lines, and added to the inhibitory effect of lysine plus threonine on the wild-type and $5MT^R$ cell lines. But neither lysine plus threonine, nor lysine, threonine and methionine together at 1.0 mM was inhibitory to the growth of the AEC^R cell line. At the higher concentration of 10 mM each lysine plus threonine totally inhibits growth of the $5MT^R$ cell line. The AEC^R cell line is not totally inhibited, by lysine plus threonine even at this concentration.

Levels of some free amino acids in the wild-type and resistant cell lines: Results of the analysis of some of the free amino acid levels in extracts from suspension cultures of the wild-type, AEC^R and $5MT^R$ cell lines are given in Table 4. The results of this analysis indicate, with the exception of alanine and methionine, a large increase in free amino acids in the AEC^R cell line relative to the levels found in the wild-type and $5MT^R$ cell lines. The AEC^R cell line showed the following approximate increases over wild-type levels; lysine 17x, histidine 10x,

Table 3. Growth of wild-type, AEC^R and $5MT^R$ Nicotiana sylvestris cells in the presence of AEC and aspartate-derived amino acids (values expressed are the mean increase in fresh weight in grams/flask after 10 days growth). Three replicates were made of each treatment.

Compound added	Cell Line		
	Wild-type	$5MT^R$	AEC^R
None	14.7	13.2	29.0
0.1 mM AEC	0.0	*	22.9
0.5 mM AEC	*	0.0	*
0.1 mM AEC + 1.0 mM Lys	11.2	*	27.7
0.5 mM AEC + 1.0 mM Lys	*	4.5	*
1.0 mM Lys	12.3	11.8	22.2
1.0 mM Thr	5.4	7.6	23.1
1.0 mM Met	3.1	7.6	14.7
Lys + Thr (1.0 mM each)	8.6	9.9	26.4
Lys + Thr + Met (1.0 mM each)	5.1	3.4	26.4
Lys + Thr (10 mM each)	*	0.0	16.7

*not tested.

None of the replicates within a treatment varied by more than 7%.

Table 4. Free amino acid levels of Nicotiana sylvestris cell lines (expressed as nmol/g fresh weight)

Amino acid	Cell Line		
	Wild-type	AEC^R	$5MT^R$
Lysine	19	334	33
Histidine	14	155	18
Aspartate	52	173	69
Glutamate	265	429	397
Alanine	543	223	259
Valine	23	186	30
Methionine	92	18	49
Isoleucine	16	62	40
Leucine	27	244	49

Free amino acids were extracted from cells grown in the absence of analogue for at least 12 generations using the methanol-chloroform-water technique of Singh et al. (1973). Duplicate cultures were analyzed and the mean values are listed. All replicates were within 10% of each other.

aspartate 3x, glutamate 1.6x, valine 8x, isoleucine 3.8x, leucine 9x. The $5MT^R$ cell line had free amino acid levels similar to those found in the parental wild-type cell line. Tryptophan is not resolved in this system. Other features of the analysis were a higher level of alanine in the wild-type, relative to the resistant cell lines, and a significant decrease in methionine in the AEC^R cell line.

Chromosome numbers: The AEC^R and $5MT^R$ cell lines used in this study have distinct and nonoverlapping differences in chromosome number. Approximately 2 years after isolation, chromosome counts of 30 metaphase figures from each of the cell lines were made. The $5MT^R$ cells showed a range of 54-178, mostly aneuploid, with a mode of 96 (octoploid, 2n=2x=24 in N. sylvestris). The variation observed was restricted to only 6 different numbers, i.e., 54(16%), 72(23%), 80(10%), 96(30%), 127(13%), and one cell with 178(3%). In contrast, all 30 AEC^R cells counted had 48 chromosomes, i.e., there was no detectable variation in number.

Discussion

While no attempt was made to determine the frequency at which spontaneous Nicotiana sylvestris variants resistant to AEC or 5MT arose, the total population of cells (about 10^7) from which single lines were isolated is within the previously reported range for higher plant biochemical variants (Widholm 1977a; Maliga 1978). Such frequency calculations use the implicit assumption that in a suspension culture placed under a "one step" lethal concentration selection, a single resistant cell can divide to produce a new culture. This assumption has not been proven experimentally, although it is conceivable that dying sensitive cells condition the culture medium. Conditioning effects have been shown to stimulate

division at lower cell population densities (Stuart and Street 1969). The resistant cell lines isolated in this study require in excess of 100 ($5MT^R$) or 1,000 (AEC^R) times as much analogue as the parent culture for total growth inhibition. Similar increases in resistance to amino acid analogues have been reported by Widholm (1976, 1978). The AEC^R cell line was able to grow without inhibition in 200 µg/ml of AEC (Fig. 1), a concentration lethal to the $5MT^R$ cell line. Conversely, the $5MT^R$ cell line showed normal growth in 30 µg/ml of 5MT (Fig. 2) which was lethal to the AEC^R cell line. Therefore, these resistant lines satisfy the requirement for nonoverlapping levels of resistance. Ideally it would be appropriate in this type of study to regenerate plants from the AEC^R and $5MT^R$ cell lines, and determine the mode of inheritance by conventional genetical analysis. After two years in culture both cell lines had lost the potential to form shoots. Such a problem is common to much of tissue culture mutant isolation (Maliga 1978), but can be overcome by using recently initiated cultures.

A secondary selection for a higher AEC-resistant line was made from AEC^R cells. The cell line selected, AEC^R1500, had increased resistance only at higher AEC concentrations, and when grown in the absence of analogue for 6 months retained about 90% of the resistance of the same cell line grown in the presence of analogue. Similar slight reductions in resistance level after long periods of nonselective growth have previously been reported by Widholm (1976).

The auxin 2,4-D is known to be an inhibitor of chlorophyll synthesis (Crafts 1975). At the concentrations used for promoting rapid, friable growth of callus and suspension cultures, little or no chlorophyll is formed, even in the presence of adequate continuous light. The bright

yellow pigmentation of the AEC^R cell line when grown in the semidarkness, and the development of a dark green color in the presence of continuous light, indicates that this line is able to overcome the inhibitory effects of 2,4-D and synthesize chlorophyll. Analysis of chlorophyll levels in protoplast-derived calli (see Chapter Three) confirm this conclusion. Although the mechanism of this resistance has not been characterized, the trait is stable in culture and may be a suitable marker in somatic cell hybridization. The two resistant cell lines also exhibit distinctive cell aggregate morphologies, but this character might be expected to change under different culture conditions, and therefore was not considered to be a suitable marker to examine in presumptive somatic cell hybrids.

In most cases the growth inhibitory effects of an amino acid analogue can be reversed by addition of the natural amino acid (Steward et al. 1958; Widholm 1976, 1977a). It is this fact that is the basic rationale for using amino acid analogues to select for amino acid overproducers. In both the wild-type and $5MT^R$ N. sylvestris cell lines the growth inhibitory effect of AEC can be reversed by 1.0 mM of lysine. However, this reversal could be due to competition for uptake into the cell rather than competition at the inhibitory site within the cell, so such studies are not conclusive. Which of these two processes is occurring might be determined by competition experiments using labelled analogue and the unlabelled corresponding natural amino acid.

The AEC^R cell line grew without inhibition in AEC or lysine plus threonine (LT) concentrations which totally inhibited growth of the $5MT^R$ cell line. One of three rice cell lines selected for their resistance to AEC also showed resistance to LT (Chaleff and Carlson 1975). Widholm (1976) isolated tobacco cell lines resistant to AEC or delta-hydroxy-

lysine (DHL) with the DHL-resistant line showing LT-resistance and the AEC-resistant line retaining LT-sensitivity. Both the rice and the tobacco resistant cell lines were shown to overproduce free lysine. Widholm (1976) suggested that this difference in resistance to LT amongst lysine analogue resistant cell lines could be due to a difference in the site of the alteration providing resistance. The AEC-resistant, LT-sensitive, cell lines could have an alteration in the feedback enzyme of the exclusive lysine portion of the pathway, while the AEC, DHL and LT-resistant cell lines could be due to an altered aspartate kinase which would affect both lysine and threonine biosynthesis. In a number of systems the inhibitory effect of LT can be partially reversed by the addition of methionine (Green and Phillips 1974; Widholm 1976). In the case of the wild-type and $5MT^R$ N. sylvestris cell lines methionine at 1.0 mM was more inhibitory than LT, and increased the inhibition of LT when combined with them. Due to the absence of any study on the aspartate pathway feedback enzymes of lysine analogue resistant cell lines, caution should be exercised in interpreting lysine overproduction or LT-resistance.

In some cases the analysis of free amino acid levels in amino acid analogue resistant cell lines is restricted to the "target" amino acid (Widholm 1976), and there have been reports of 2- to 11-fold increases in the level of free lysine in cell lines resistant to AEC (Chaleff and Carlson 1975; Widholm 1976, 1978). It has been demonstrated that increases in free tryptophan in 5MT-resistant, and phenylalanine in PFP-resistant cell lines, are due to altered feedback enzymes giving relaxed control (Widholm 1972; Palmer and Widholm 1975). However, in the N. sylvestris AEC^R cell line there was not only a 17-fold increase in free lysine over the level in the wild-type, but also large increases in other

free amino acids, some of which are not derived from aspartate. Similar results have been reported by Chaleff and Carlson (1975) for rice AEC-resistant cell lines. These results make it difficult to determine the significance of free lysine increases in lysine analogue resistant cell lines, and studies on the feedback properties of the enzymes controlling lysine biosynthesis are needed.

The $5MT^R$ cell line has both a slightly higher level of free lysine (Table 4) and less AEC-sensitivity than the wild-type parent culture (Fig. 1). A comparable slight decrease in AEC sensitivity in a carrot cell line selected for resistance to ethionine, which also showed a small increase in free lysine has been reported by Widholm (1978). While it is apparent that changes in the levels of amino acids other than the "target" amino acid do occur, it is not clear whether these cause changes in resistance to other analogues.

The mechanism of resistance in the AEC^R cell line is not yet known, but if it is due to the higher level of free lysine a number of biochemical alterations could be responsible:

a) An altered aspartate kinase resistant to feedback inhibition by lysine could confer resistance to AEC-resistant cells. Such a phenomenon has been observed by Widholm (1972a,b) with anthranilate synthetase from 5MT-resistant cell lines of tobacco and carrot.

b) The alteration need not be a mutation in a gene coding for aspartate kinase, but could be regulatory in nature. Carlson and Widholm (1978) have described the separation of feedback sensitive and resistant forms of anthranilate synthase from both 5MT-resistant and susceptible cells, with a predominance of the corresponding form in the respective cell type. Two forms of aspartate kinase (lysine sensitive and threonine

sensitive) have also been identified (Cheshire and Miflin 1975; Mazelis et al. 1977; Matthews and Widholm 1978), and proportions of the two are different in cultured cells and in whole roots. Resistance to feedback inhibition by lysine and inhibition by AEC could arise by a reduction or lack of the lysine-sensitive form in culture.

c) The branch point enzyme in lysine biosynthesis, dihydrodipicolinic acid synthetase, is also feedback inhibited by lysine (Cheshire and Miflin 1975; Mazelis et al. 1977; Matthews and Widholm 1978). Therefore, an altered dihydropicolinic acid synthetase resistant to feedback inhibition might provide resistance to AEC. Only one form of this enzyme was found in extracts from carrot suspension cultures and whole roots (Matthews and Widholm 1978).

d) In cultured mouse cells resistance to the 4-amino analogue of folic acid, methotrexate, results from a higher content of dihydrofolate reductase, and a corresponding increase in the number of copies of the gene coding for this enzyme (Schimke et al. 1978). However, to date no such resistance related gene amplification has been demonstrated in plant cells.

If the AEC resistance were not due to increased free lysine diluting the analogue within the cell a number of possible mechanisms of resistance exist. One possibility is a reduction in the uptake of the analogue. Bright and Miflin (1978) have reported the isolation of barley plants resistant to AEC which have a reduced uptake of labelled lysine. This character is inherited as a recessive. Another possible mechanism is a reduction in incorporation of the analogue into proteins due to an altered amino-acyl-synthetase which could distinguish between the analogue and the natural amino acid.

Hybrid cells selected after protoplast fusion might be expected to have a higher chromosome number than the parents. Therefore, chromosome counts were made on the AEC^R and $5MT^R$ cell lines. All cells examined in the AEC^R cell line had 48 chromosomes (tetraploid), whereas the $5MT^R$ cell line had a range of numbers from 54 to 178, with a mode of 96. Modification of chromosome numbers with the resulting polyploidy and aneuploidy in plant tissue cultures is well documented (Sunderland 1973; D'Amato 1978). But there are also examples of stability with all cells having the same number of chromosomes, similar to that shown in the AEC^R cell line (Kao et al. 1970).

Because the AEC^R and $5MT^R$ cell lines were isolated specifically for protoplast fusion and the attempted selection of somatic hybrids, the criteria of stability applied is that they retain this resistance after protoplast isolation and culture. Experiments to test this are described in Chapter Two. No stability tests on the AEC^R or $5MT^R$ cell lines were made in suspension culture.

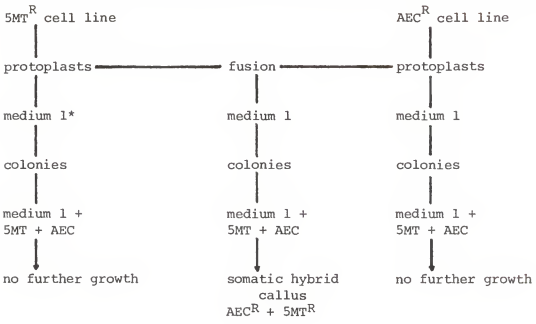
CHAPTER THREE

SELECTION OF SOMATIC CELL HYBRIDS

Introduction

In order to test the hypothesis that AEC^R + $5MT^R$ somatic cell hybrids of Nicotiana sylvestris can be selected after protoplast fusion of the parental lines, it was necessary to determine appropriate culture conditions for the induction of callus formation from protoplasts of the AEC^R and/or the $5MT^R$ suspension culture cell lines. In this intraspecific somatic hybridization where there were limited nonselected markers for hybrid identification, it was necessary to have control experiments which would test possible alternative explanations for the appearance of double-resistant calli. Such calli should appear only after heterokaryotic protoplast fusion. Control experiments were designed to test for the existence of double resistance with individual cell lines, double resistance due to cross-feeding, and the possibility that polyploidy resulting from homokaryotic fusion might increase amino acid analogue resistance. The successful selection of AEC^R + $5MT^R$ somatic cell hybrids provided an opportunity to analyze the mode of AEC and 5MT resistance expression. Partial analysis was also made of the expression of the AEC^R chlorophyll synthesis character in the hybrid cells. The selection scheme used in this study is outlined in Fig. 6.

Fig. 6. Somatic hybrid selection scheme using amino acid analogue-resistant cell lines.



*protoplast culture medium without amino acid analogues.

Materials and Methods

Protoplast isolation and culture: All procedures were carried out under aseptic conditions in a laminar flow cabinet and all media, solutions, centrifuge tubes etc., were autoclaved at 15 psi for 15 min. Protoplasts of the AEC^R and $5MT^R$ cell lines were isolated and cultured in the medium of Nagy and Maliga (1976), containing 250 mg/l glucose, 0.4 mg/l 2,4-D, 0.03 mg/l kinetin, and 0.4 M sucrose as the osmoticum. Cells of four-day old suspension cultures were collected on a 100 µm stainless steel filter and added to 10 ml of protoplast isolation medium: 6% Cellulysin (Calbiochem), 4% Driselase (Kyowa Hakko), 1% Macerozyme (Yakult Biochemical), pH 5.6, to give a final volume of 20 ml/50 ml Erlenmeyer flask. Flasks were incubated at 30°C for 5-6 hours at 80 strokes/min in a water-bath shaker. Coarse debris was removed by filtering the protoplasts through 100 µm and 50 µm stainless steel filters. The protoplasts floated in the culture medium containing 0.4 M sucrose and were washed four times by centrifugation at 300 x g for 3 min in this medium. Each time the floating protoplasts were transferred to a new centrifuge tube using a Pasteur pipet. Finally the floating protoplasts were collected and the number adjusted to ca 10^6/ml and mixed with an equal volume of 1.2% agar medium maintained at 40°C to give final agar and protoplast concentrations of 0.6% and 5 x 10^5/ml, respectively. One ml of this protoplast suspension was overlayed on 5 ml of medium previously solidified with 1% agar in a 50 x 9 mm sterile Falcon plastic Petri dish. After the protoplast containing layer had solidified the plates were sealed with Parafilm, and incubated at 28°C in continuous light of 500 lux, inside a tight fitting plastic box to maintain humidity.

Protoplast fusion: For heterokaryotic fusion AEC^R and $5MT^R$ protoplasts were mixed 1:1 after washing. Fusion was induced by a modification of the method of Kao (1977). To 400 µl of protoplast suspension in a Falcon plastic Petri dish was added 600 µl of polyethylene glycol solution (PEG), as described by Kao (1977), to give a final PEG concentration of 30%. A 22 x 22 mm glass cover slip was placed on top of the droplet so that the floating protoplasts adhered to its underside. After 25 min at room temperature, 600 µl of a high calcium-high pH solution (50 mM $CaCl_2 \cdot 2H_2O$, 50 mM Na-glycine buffer, 0.4 M sucrose) was added slowly. After 15 min, 1 ml of the protoplast culture medium was added. About 75% of the solution under the coverslip was carefully removed, and the protoplasts were gently washed an additional 5 times before being shaken free from the underside of the coverslip. The collected protoplasts were then washed twice by centrifugation, adjusted to ca 10^6/ml and plated. Controls included (a) plating 1:1 mixed AEC^R and $5MT^R$ protoplasts without fusion, and (b) fusing AEC^R + AEC^R protoplasts and $5MT^R$ + $5MT^R$ protoplasts separately, washing, and then mixing 1:1 before plating.

Somatic cell hybrid selection: After 35-60 days of culture dividing protoplasts had formed small calli of approximately 50-100 cells. The top soft agar layer containing the calli was cut into 1 cm wide strips, which were separated from the underlying hard agar with a spatula, and transferred to 5 ml of the selection medium plated in a 50 x 9 mm Petri dish. This medium was the same as the protoplast culture medium but with reduced osmoticum (0.2 M sucrose) and containing 200 µg/ml of AEC and 30 µg/ml of 5MT. Those calli which survived and grew were transferred after ca 20 days to the selection culture for only one 2 week culture

period, and were thereafter grown in the absence of the analogues. Selection and maintenance of the somatic hybrid calli was made in ca 2,000 lux of continuous light.

Resistance levels of parental and hybrid calli, and the stability of double resistance in hybrid calli, were determined by transferring small callus pieces (30 mg) to agar medium (5 ml per 50 x 9 mm Petri dish), incubating for 14 days, and recording the fresh weight increase. Similarly, auxin-dependence of growth was determined by transferring 30 mg callus pieces to agar medium lacking 2,4-D, culturing for one week, recording fresh weight increase of some, and transferring others to the same medium for a further one week culture. This was continued (3 sub-cultures) until fresh weight increase ceased. The values given in all these experiments represent the means of 16 replicates.

Chlorophyll determinations were made on extracts of parental protoplast-derived and hybrid calli using the method of Arnon (1949), after 14 days of growth. Two grams fresh weight of callus was homogenized in a Pyrex 15 ml glass tissue grinder with 3 ml of 80% acetone saturated with $MgCO_3$. The volume was made up to 10 ml and the solution filtered through a 0.8 µm Millipore filter, then centrifuged for 5 mins at 12,000 x g. All procedures were at 4°C. Absorbance readings were made at 663 nm, 652 nm, and 645 nm, using a Beckman 25 spectrophotometer. An absorbance scan was also made from 750 nm to 350 nm.

Chromosome counts were made of the somatic hybrid calli using the procedure outlined in Chapter Two.

Free amino acid were extracted from protoplast-derived parental and hybrid calli, after 14 days of growth, using the technique given in Chapter Two, with the following additional procedures. To 1 ml of the

final sample dissolved in 0.01 N HCl, 9 ml of 100% ethanol was added. After a period of precipitation at 4°C the sample was centrifuged for 30 min at 7,700 x g. Five ml of the supernatant was evaporated in a Sarvant Bio dryer and the residue dissolved in 10 ml of 0.01 N HCl. Analysis was as described in Chapter One. Free tryptophan levels were determined using a 5x concentrated sample, analyzed separately, and using 99 nm tryptophan as a standard.

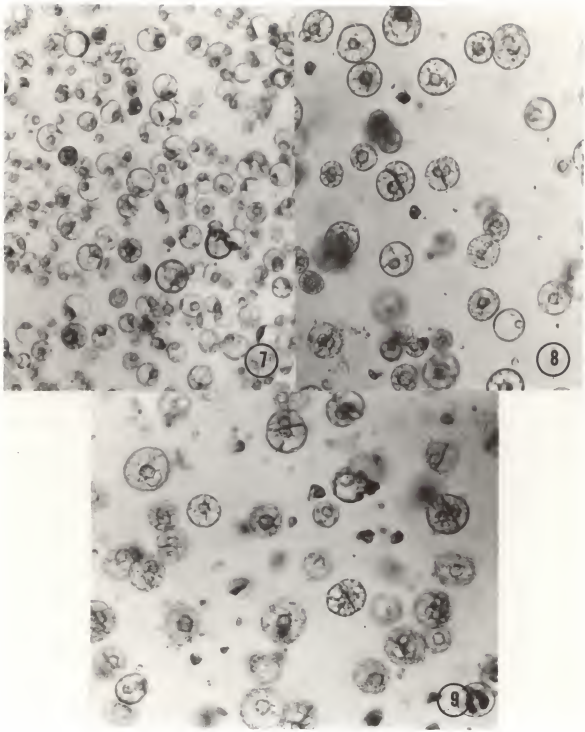
Results

Protoplast isolation and culture: Protoplast isolation from the AEC^R cell line was very effective, with almost all the cells forming protoplasts after 5 hours of incubation in the isolation medium. Digestion of cell walls in $5MT^R$ cells was not always complete, and about 10% of the original cells were not converted to protoplasts at the end of the 5-6 hour incubation period. The procedure of washing by flotation in the culture medium containing sucrose resulted in a very clean preparation of protoplasts devoid of cellular debris and cells (Fig. 7). Some of the AEC^R protoplasts divided after 3 days in culture (Fig. 8), and 40-70% had divided at least once 10 days after plating (Fig. 9). Calli of up to about 50 cells were formed after 20 days in culture. The AEC^R protoplasts did not change significantly in their shape or size prior to or during the first few divisions. In contrast to the AEC^R protoplasts, only 5-10% of the $5MT^R$ protoplasts divided, and very few (0-4 per plate) showed sustained divisions and formed calli. The $5MT^R$ protoplasts expanded and changed shape prior to dividing. When mixed with the AEC^R protoplasts in control experiments, about 1-5% of the $5MT^R$ protoplasts were able to form calli.

Fig. 7. AEC^R protoplast preparation prior to plating.

Fig. 8. Division of AEC^R protoplasts after 3 days in culture.

Fig. 9. Plating efficiency of AEC^R protoplasts after 10 days in culture. Note the division with little change in shape or size.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This was inferred from the difference in pigmentation and cell aggregate morphology between the AEC^R and $5MT^R$ protoplast-derived calli.

Resistance of AEC^R protoplast-derived cells to AEC: A major requirement of the proposed selection system (Fig. 6) is that the resistant cells retain resistance after protoplast isolation and culture, i.e., cloning. AEC^R protoplast-derived calli were transferred to medium containing AEC or 5MT after growing in the absence of analogue for 20 days. Growth of these protoplast-derived calli was not inhibited on 200 µg/ml of AEC, and even cell masses containing 4-6 cells were observed to continue dividing and form larger calli (Table 5). A 5MT concentration of 20 µg/ml was lethal to the AEC^R calli. Examination of the stability of 5MT-resistance in $5MT^R$ protoplast-derived calli was not possible due to their low plating efficiency.

Protoplast fusion: Attempts to fuse AEC^R and $5MT^R$ protoplasts floating on the surface of culture medium droplets, with PEG, resulted in an interaction between the protoplast and the liquid-air interface, and after 5-10 min the protoplasts lysed. In order to overcome this problem a glass coverslip was placed on the top of the droplet immediately after the addition of PEG. Protoplasts adhered to each other and to the underside of the coverslip (Fig. 9). During dilution of the PEG with the high Ca^{++}- high pH solution many of the protoplasts adhering to each other separated, but some were observed to fuse (Fig. 10). After protoplast isolation about 1-2% of the protoplasts were binucleate. This proportion appeared to increase after fusion treatment (Fig. 11). However, in the absence of any morphological markers in the protoplasts it was not possible to discern whether AEC^R + $5MT^R$ heterokaryons had been formed.

Table 5. Resistance/susceptibility of AEC^R protoplast-derived calli to S-2-aminoethyl-cysteine or 5-methyl-tryptophan (tested after 20 days growth in the absence of analogue)

Analogue	Concentration (µg/ml) 0	5	10	15	20	25	30	40	200
AEC	+++	+++	+++	+++	+++	+++	+++	+++	+++
5MT	+++	++	++	+	-	-	-	-	-

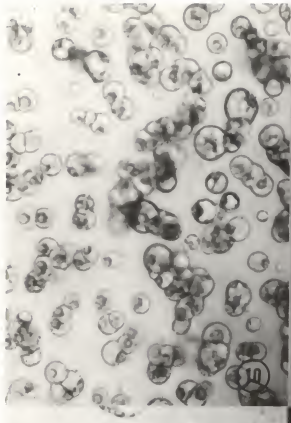
+++ no inhibition of growth
++ more than 50% inhibition of growth
+ less than 1% growing, only 1-3 calli surviving per plate
- no growth

Fig. 10. Polyethylene glycol (PEG) induced agglutination of a mixture of AEC^R and $5MT^R$ protoplasts.

Fig. 11. Early stages of fusion after the addition of the high Ca-high pH solution.

Fig. 12. Binucleate protoplasts after the fusion procedure.

Fig. 13. AEC^R + $5MT^R$ somatic hybrid callus selected on double analogue medium, 14 days after transfer.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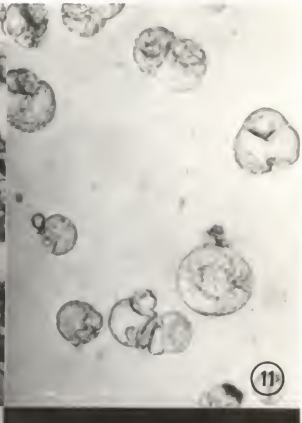
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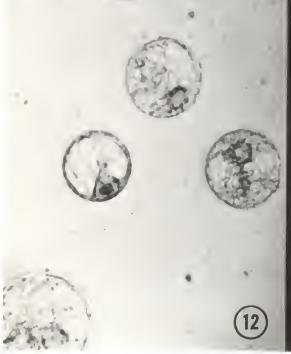
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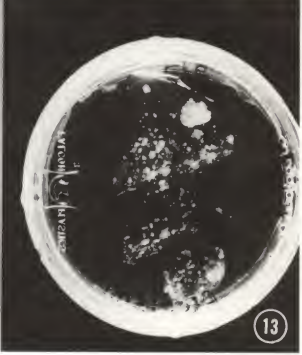
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Selection of somatic cell hybrids: Protoplasts in both fusion and control experiments were grown for at least 35 days in the absence of analogues, prior to being transferred to the selection medium. Of the 975 calli derived from three separate fusion experiments, 8 calli (0.8%) were able to grow without inhibition on the double-analogue selection medium (Table 6 and Fig. 13). These calli which all grew rapidly when subcultured onto the selection medium, were designated H1 through H8, and were characterized further.

Two types of control experiments were performed. First a total of 1.8×10^4 calli from mixed AEC^R and $5MT^R$ protoplasts were placed on the selection medium, but none survived (Table 6). In order to test the possibility that larger calli might overcome the selection, these calli were grown for a longer period (45 or 60 days), prior to selection, than fusion experiments. The plating density in these experiments was such that AEC^R and $5MT^R$ calli were observed immediately adjacent to each other, but these died at the same time as other control calli when placed on the selective medium. Secondly, AEC^R and $5MT^R$ protoplasts were fused separately (homokaryotic fusion), washed to prevent any further fusion, and then mixed 1:1 before plating. A total of 3×10^3 calli obtained from these control experiments were placed on the selection medium but none survived (Table 6).

Characterization of somatic hybrid cell lines: Somatic hybrid calli were transferred to the selection medium only once after isolation, and were thereafter grown in the absence of analogues. Protoplast-derived calli of the parental cell lines were also maintained in the absence of analogues. Stability of double-resistance in some of the hybrids and

Table 6. Fusion and control experiments with AEC^R and $5MT^R$ protoplast-derived calli

	Period of Culture Prior to Selection (days)	Number of Colonies Screened	Number of Calli Selected in a Double-Analogue Medium
Control Experiments			
Mixed AEC^R and $5MT^R$ protoplasts	60	5×10^3	0
Mixed AEC^R and $5MT^R$ protoplasts	45	10^4	0
AEC^R amd $5MT^R$ protoplasts fused separately then mixed 1:1	35	3×10^3	0
Fusion Experiments			
AEC^R + $5MT^R$ protoplasts			
1.	35	656	6
2.	35	236	1
3.	35	83	1

confirmation of the inability of the parental calli to grow on the selection medium, was tested 3 and 6 months after isolation of the hybrids. Maintenance of double-resistance in H1, H3, and H5 calli, versus lack of growth of AEC^R or $5MT^R$ calli, after 3 months, is illustrated by Fig. 14 a, b. Fresh weight increases of AEC^R, $5MT^R$, H1, H3, and H5 calli grown in the presence or absence of 200 µg/ml AEC + 30 µg/ml 5MT were determined after 6 months growth in the absence of the analogues (Table 7). These results indicate that the hybrids retained double-resistance when grown for long periods in the absence of selection and were therefore stable for this trait.

Three of the selected calli (H1, H3, and H5) were studied to determine their levels of resistance to AEC and 5MT, compared with the parental cell lines. All three hybrids demonstrated AEC-resistance curves very similar to the AEC^R parent; H3 and H5 are shown in Fig. 15. The H1 and H3 calli have a 5MT-resistance level similar to the $5MT^R$ parent at 30 µg/ml, but show a distinctly greater sensitivity at higher levels of the analogue (Fig. 18). A similar intermediate level of resistance was shown by H5 calli.

As outlined in Chapter One the parental cell lines used in this study, AEC^R and $5MT^R$, have distinct and nonoverlapping differences in chromosome number. Suspension cells of the AEC^R line have 48 chromosomes, with no detectable variation in number (Table 8). The $5MT^R$ suspension cells exhibited a 54-178 range in number, with a mode of 96. Chromosome numbers in some of the somatic cell hybrids, H1, H2, H3, and H5, were determined in callus cells 3-4 months after isolation. Three hybrids, H1(216), H3(223), and H5(192), had numbers greater than either parental

Table 7. Stability of double-resistance in AEC^R + $5MT^R$ somatic hybrid calli. Each value given is the mean of 12 replicates (fresh weight increase in mg/callus)

Cell Line	Analogues	
	None	200 µg/ml AEC + 30 µg/ml 5MT
AEC^R	494.5	0.0
$5MT^R$	221.4	0.0
H-1	427.5	228.1
H-3	386.6	305.0
H-5	424.3	310.0

Fig. 14a. Parental and H1, H3, and H5 hybrid calli on medium containing 200 ug/ml AEC and 30 ug/ml 5MT, at the beginning of the culture period.

Fig. 14b. Growth of hybrid calli on the double analogue medium after 14 days of culture. Parental cells were observed to be dead. All calli had been grown in the absence of analogues for 3 months prior to testing for stability of double resistance.

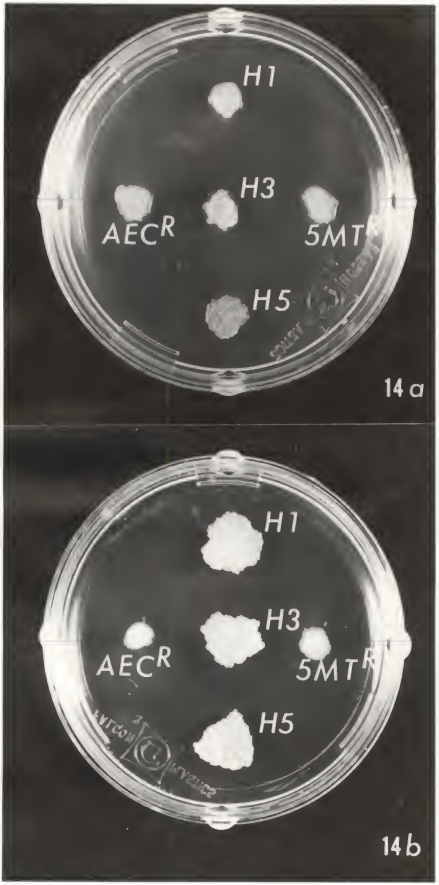

H1
H3
AEC^R
5MT^R
H5
14 a
H1
H3
AEC^R
5MT^R
H5
14 b

Fig. 15. Effect of S-aminoethyl-cysteine on the growth of AEC^R (o), $5MT^R$ (●), and hybrid H3 (□) and H5 (■) cells. Values are the increase in fresh weight given as a percentage of the same line grown in the absence of the analogue. Period of growth 14 days.

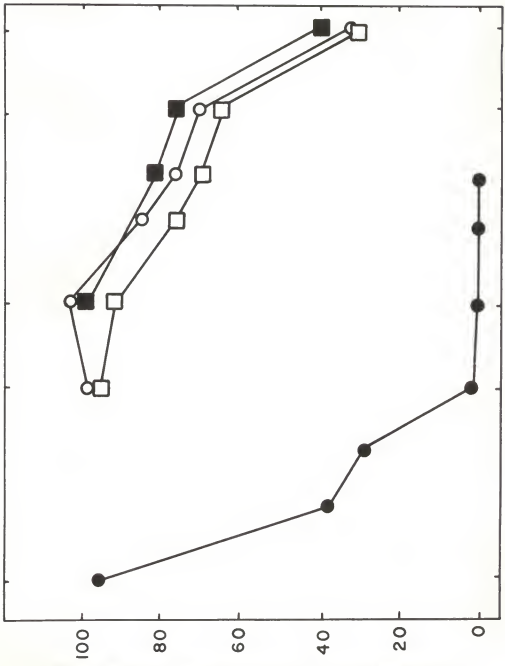

FRESH WEIGHT INCREASE (% OF CONTROL)
0
20
40
60
80
100
10
50
100
500
1000
S-AMINOETHYL-L-CYSTEINE (μg/ml)

Fig. 16. Effect of 5-methyl-tryptophan on the growth of $5MT^R$ (●), AEC^R (o), and hybrid H3 (□) and H1 (Δ) cells. Values are the increase in fresh weight given as a percentage of the same line grown in the absence of the analogue. Period of growth 14 days.

FRESH WEIGHT INCREASE (% OF CONTROL)
0
20
40
60
80
100
10
50
100
500
1000
5-METHYL TRYPTOPHAN (ug/ml)

Table 8. Chromosome numbers of parental and hybrid cell lines

Cell Line	Number of Cells Observed	Chromosome Number	
		Mode	Range
AEC^R	30	48	none
$5MT^R$	30	96	54-178
H1	3	216	*
H2	1	175	*
H3	1	223	*
H5	6	192	*

*Number of cells observed insufficient to preclude range, see text.

cell line. A metaphase plate of a H3 callus cell is shown in Fig. 17, and that of a $5MT^R$ suspension cell with 96 chromosomes in Fig. 18.

All the hybrid calli had a light green pigmentation, intermediate between the darker green AEC^R and the white $5MT^R$ protoplast-derived calli. A visual comparison of the parents and H2 calli is shown in Fig. 19. This pigmentation difference was reflected in the results of total chlorophyll determinations (total chlorophyll mg/g fresh weight: AEC^R=0.213, H2=0.106, $5MT^R$=0.0). In order to determine if the photosynthetic pigments were qualitatively similar in the AEC^R and H2 calli, an absorbance scan from 350 to 750 nm was made on the same 80% acetone extracts as were used for chlorophyll determination (Fig. 20). There is no substantial qualitative difference in the absorbance spectra of the AEC^R and H2 pigments, just a difference in amount.

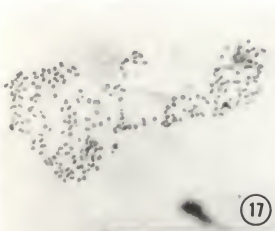
It has been reported that some tobacco cell lines selected for resistance to 5MT are also auxin-autotrophic, i.e., do not require an auxin for sustained growth (Widholm 1977b). This characteristic might influence the selection system used in this study, therefore, AEC^R and $5MT^R$ parental calli and H1, H3, and H5 calli were tested for growth in the absence of 2,4-D (Table 9). All calli showed a decrease in growth rate when transferred to medium lacking 2,4-D, and by the third subculture in this medium there was no fresh weight increase and the calli were dry and brown in appearance. It was concluded that all the cell lines tested were auxin-dependent for growth.

The levels of free amino acids extracted from AEC^R, $5MT^R$, and hybrid H1, H3, and H5 calli are given in Table 10. In most cases the $5MT^R$ cell line had a higher concentration of the amino acid examined than any of

Fig. 17. Mitosis in a callus cell of H3. x 950.

Fig. 18. Mitosis in a suspension cell of the $5MT^R$ cell line. x 1250.

Fig. 19. Pigmentation differences between the parental protoplast-derived calli, and an intermediate expression in H2 hybrid calli.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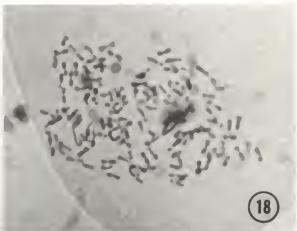
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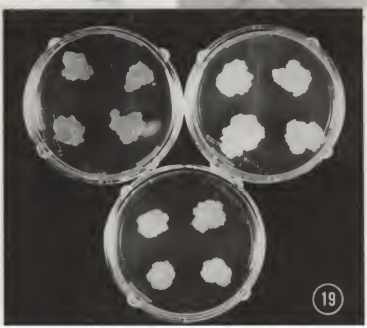
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Fig. 20. Absorbance of extracted photosynthetic pigments of AEC^R and H-2 cells in the wavelength range 350-750 nm.

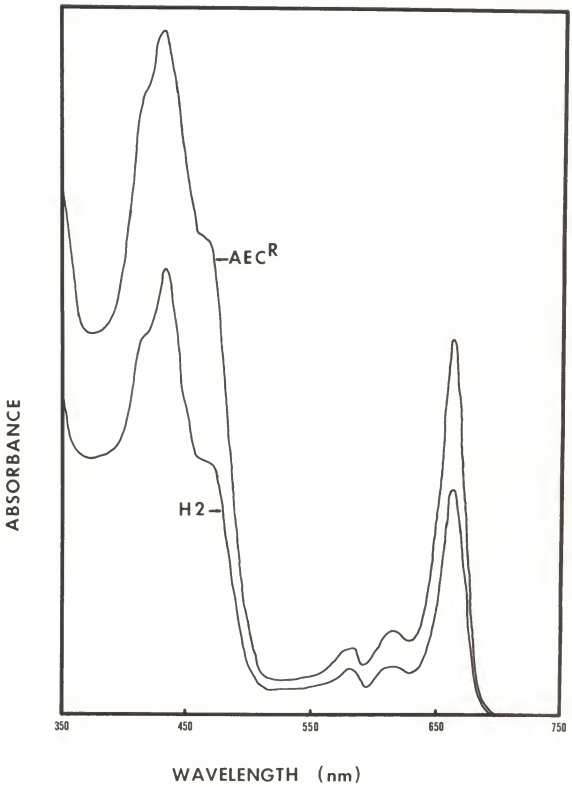
AEC^R
H2
ABSORBANCE
350
450
550
650
750
WAVELENGTH (nm)

Table 9. Growth of parental and hybrid cell lines during successive passages on 2,4-D free medium. Values are the mean fresh weight increases of 16 replicates ± standard deviation.

Cell line	1st passage		2nd passage		3rd passage	
	-2,4D	+2,4D	-2,4D	+2,4D	-2,4D	+2,4D
AEC^R	71±31	140±15	16±19	125±33	2±4	135±26
$5MT^R$	75±23	122±33	15±8	118±21	2±4	134±19
H-1	90±19	124±22	20±5	121±29	0±2	144±17
H-3	119±24	214±49	18±10	250±22	0±7	201±12
H-5	155±29	230±58	20±19	217±44	2±4	200±37

Table 10. Free amino acid levels of Nicotiana sylvestris parental and somatic cell hybrid calli (expressed as nmol/g fresh weight).

Amino acid	Cell Line				
	$5MT^R$	AEC^R	H1	H3	H5
Lysine	34.5	25.8	30.1	27.6	22.4
Histidine	42.8	105.2	15.1	*	16.5
Arginine	127.4	*	*	*	21.5
Aspartate	157.4	45.9	36.5	28.6	87.2
Glutamate	411.4	261.6	254.7	35.0	290.5
Glycine	115.2	46.7	84.1	56.7	44.9
Alanine	649.7	191.1	338.6	139.4	256.9
Valine	63.3	42.5	56.7	47.8	26.3
Methionine	42.0	22.6	27.6	47.8	31.4
Isoleucine	65.7	21.5	27.6	29.9	31.4
Leucine	93.7	38.7	42.8	45.3	57.2
Tyrosine	78.3	17.9	27.2	21.2	27.2
Phenylalanine	64.8	*	21.4	17.6	22.7
Tryptophan	201.8	22.4	74.2	66.4	54.5

*Too dilute in the sample to be resolved. Free amino acids were extracted from parental and hybrid cells grown in the absence of analogue for 6 months using a modification of the technique of Singh et al. (1973), see Methods and Materials. Duplicate cultures were analyzed and the mean values are listed. All replicates were within 10% of each other.

the other cell lines. However, differences between the cell lines in free lysine content were at most only 1.5-fold. Levels of most free amino acids examined were similar in the AEC^R, H1, H3, and H5 cell lines. The exception to these patterns was the about 9-fold increase in tryptophan in the $5MT^R$ line over that level exhibited by the AEC^R cell line. Hybrid cells examined had a level of tryptophan intermediate between that of the parental cell lines.

Discussion

The conclusion that the 8 cell lines selected after heterokaryotic fusion treatment are somatic hybrids relies on their resistance to both AEC and 5MT (Figs. 15, 16, and Table 7). Previously intraspecific somatic hybridization by protoplast fusion in Nicotiana tabacum and Datura innoxia has been demonstrated definitively by segregation of complementary mutations for chlorophyll-deficiency in the progeny (Melchers and Labib 1974; Scheider 1978b). Such segregation analysis is not applicable to this study because of the absence of plant regeneration in hybrid and parental cell lines. Therefore, considerable emphasis has been placed on designing control experiments which eliminate other possible explanations for the occurrence of double-resistant cell lines from fusion experiments.

Of the large number of calli (1.5×10^6), from mixed unfused AEC^R and $5MT^R$ protoplasts, placed on the selection medium, none survived. This shows that colony growth on the selection medium is dependent on protoplast fusion and could not be due to metabolic cooperation between the AEC^R and $5MT^R$ cells (cross-feeding). Furthermore, the existence of double-resistant cells within one of the parental resistant cell lines at a frequency higher than one cell in 1.8×10^4, is ruled out (Table 6). Appearance of new

resistants at a frequency equivalent to that of the hybrid selection (0.8%) is very unlikely, and would be contrary to my experience with Nicotiana sylvestris and other data in the literature (Widholm 1977a; Maliga 1978). Mixed AEC^R and $5MT^R$ unfused control calli were also cultured for a longer period prior to selection than those from fusion experiments, in order to eliminate any possible effect of a larger callus size. The possibility that an increase in ploidy level due to homokaryotic fusion might change the resistance expression was similarly eliminated by fusing the two parental lines separately, washing to stop any further fusion, and then mixing the protoplasts. A total of 3×10^3 calli from this type of control experiment were placed on the selection medium but none survived (Table 6). After completion of all the control experiments it was concluded that the selected double-resistant calli were the result of AEC^R + $5MT^R$ heterokaryotic fusions.

Characterization experiments on hybrid calli were carried out primarily with the H1, H3, and H5 cell lines. These lines exhibited stability of double-resistance after growth for 3-6 months in the absence of both analogues (Figs. 14a, b, Table 7). This stability of double-resistance, combined with the lack of cross-feeding in control experiments, excludes the possibility that H1, H3, and H5 calli are chimeras of AEC^R and $5MT^R$ cells.

Resistance of H1, H3, and H5 calli to AEC, is very similar to that of the AEC^R line (Fig. 15). Similar demonstrations of drug resistance expression in mammalian somatic cell hybrids have been interpreted as evidence of dominance of the resistance phenotype (Harris 1973; Baker et al. 1974). Resistance of the hybrid calli to 5MT is at an intermediate level between that of the $5MT^R$ and AEC^R cell lines (Fig. 16). This could

be characterized as semidominant expression of the 5MT-resistance. Some types of mutant cell lines selected in plant tissue culture may not regenerate due to the nature of the mutation (Muller and Grafe 1978). In such cases genetic analysis of the character in somatic hybrids may be an alternative to sexual analysis.

Of the four hybrids examined, three (H1, H3, and H5) had numbers greater than either parent, and one (H2) a number within the $5MT^R$ range but not previously observed (Table 8). These chromosome numbers can be explained in terms of additions of the AEC^R and $5MT^R$ parents, and their subsequent modifications, known to occur in tissue culture cells (Sunderland 1973).

All of the hybrids had levels of pigmentation intermediate between the AEC^R and $5MT^R$ cell lines. One hybrid cell line (H2) was studied in more detail in order to quantify and partially qualify this unselected phenotypic expression. Determination of the total chlorophyll content showed the hybrid to have about half the level of the AEC^R cell line. The absorption spectra of photosynthetic pigments are the same in extracts from the AEC^R and H2 cells (Fig. 20). It can be concluded that there is no major qualitative difference in the pigments examined, just a difference in amount. In the presence of a high 2,4-D (auxin) to cytokinin ratio, rapidly dividing dicotyledonous plant cells grown in culture, are in a de-differentiated state, with inhibition of proplastid development, and consequentially lack chlorophyll-containing chloroplasts. Bornman (1974) has suggested that either a stimulus which affects the differentiation of chloroplasts from proplastids is lacking, or is blocked in these cells. the AEC^R cell line is able to overcome this normal inhibition and may be a suitable material for studying the control of proplastid to chloroplast development.

Some of the 5MT-resistant cell lines previously isolated proliferate in the absence of an auxin (auxin-autotrophy, Sung 1975; Widholm 1977b). In order to determine whether this auxin-independence was a factor in the AEC^R + $5MT^R$ hybrid cell selection, AEC^R, $5MT^R$, H1, H3, and H5 calli were tested by sequential transfer on 2,4-D-free medium (Table 9). Growth rates of the calli in 2,4-D-free medium were more variable than in the presence of 2,4-D, but by the third passage on 2,4-D-free medium growth had almost stopped in all of the cell lines. It was concluded that all the cell lines tested were dependent on 2,4-D for growth, and that auxin-autotrophy was not a factor in the success of the selection scheme.

Results of the free amino acid analysis of extracts from parental and hybrid calli demonstrate, as was shown in Chapter One, that it is not possible to make conclusions about free amino acid overproduction based solely on examination of the "target" amino acid levels. In this case the levels of almost all amino acids examined were higher in the $5MT^R$ cell line than in the AEC^R and hybrid cell lines. This means that the small difference between the free lysine content of the $5MT^R$ cells and that of the other cell lines is not significant. Apparently neither the AEC^R nor the hybrid cell lines accumulate free lysine. In the absence of an examination of the aspartate pathway feedback control enzymes in the AEC^R, and the hybrid cell lines, it is not possible to discount free lysine overproduction as the AEC-resistance mechanism. Miflin (1975) reported the isolation of lysine analogue-resistant carrot cell lines which did not accumulate free lysine, but contained aminoadipic acid and pipecolic acid, which are products of lysine degradation. AEC has been shown to be inhibitory to the activity of aspartate kinase extracted from carrot cell suspensions (Davies and Miflin 1977), and this and the

AEC^R resistance to lysine plus threonine, makes this enzyme a logical choice to examine first when looking for the AEC^R cell line mechanism of resistance.

Despite the overall increase in free amino acid levels in the $5MT^R$ cells there is a significant increase in free tryptophan in the $5MT^R$ line compared with the level in the AEC^R cell line (Table 10). The hybrid cell lines exhibited tryptophan levels intermediate between the levels found in the parental cells. While no definite conclusion can be reached without examination of the feedback enzyme anthranilate synthetase, in the parental and hybrid cell lines, it appears that the $5MT^R$ cell line overproduces tryptophan, and this overproduction is partially expressed in the hybrids. Data in the literature suggest a possible explanation for this partial expression of tryptophan overproduction and the semidominance of 5MT-resistance in AEC^R + $5MT^R$ hybrid cells. Carlson and Widholm (1978) have examined anthranilate synthetase from 5MT-susceptible and -resistant lines of potato, and shown the 5MT-resistant line to be a tryptophan overproducer. Separate 5MT-resistant and -sensitive forms were found in both cell lines, with the resistant form predominating in the resistant line, and the sensitive form in the susceptible line. One possible explanation is that the 5MT-resistance may be due to an altered regulatory element which enhances synthesis of the 5MT-resistant form of anthranilate synthetase in the 5MT-resistant cell line. A hybrid between such a resistant cell and a 5MT-sensitive cell might be expected to have similar synthesis of both forms of the enzyme, and therefore, intermediate levels of resistance and tryptophan overproduction.

In mammalian somatic cell genetics drug resistant or enzyme-deficient mutants have been used extensively to isolate somatic cell hybrids. To

date, only a limited application of these approaches has been made in higher plant somatic hybridization. Kanamycin resistance has been used as part of a selection screen to obtain Nicotiana sylvestris + N. knightiana somatic hybrids (Maliga et al. 1977). Shoot induction capability, deficient in the kanamycin resistant N. sylvestris cell line, was restored in the hybrids, and sexual genetical analysis of the kanamycin resistance may now be possible. Recently Glimelius et al. (1978) were able to select wild-type Nicotiana tabacum somatic hybrids after fusing two complementing, nitrate reductase deficient mutant cell lines. Both mutants used were auxotrophic for reduced nitrogen (e.g., amino acids) due to deficiency of nitrate reductase, and unable to grow on media containing nitrate as sole nitrogen source. Hybrids were selected from a mixed population of parental types and hybrid cells by means of their ability to grow on nitrate as sole nitrogen source. The hybrids had regained nitrate reductase activity and were able to differentiate into shoots, both characteristics lacking in the mutant cell lines. Controls similar to those in this study were used to eliminate back mutation and cross-feeding as possible explanations for the occurrence of cell lines utilizing nitrate. It is noteworthy that hybrids were only obtained if both parental and hybrid protoplasts were allowed to regenerate into cells and form cell clusters by a culture period in amino acid medium prior to being placed under selection. It was suggested that this initial period of growth under nonselective conditions may be necessary in order to achieve a sufficiently high density of metabolically active cells and to allow the hybrid cells to synthesize nitrate reductase. In most cases it is necessary to have a certain protoplast density in order to obtain division of higher plant protoplasts.

The lack of a culture period prior to selection may explain the lack of success in the attempt to use resistant and auxin-autotrophic cell lines for selection of somatic hybrids (Chourey and Widholm 1978). Auxin-autotrophic carrot or tobacco protoplasts were fused with AEC-resistant or tobacco threonine-resistant protoplasts and plated directly on the appropriate selection medium (Chourey, pers. commun.). In none of these combinations, intraspecific or intergeneric, were any hybrid calli obtained. Recently, a selection procedure for mammalian cells has been developed by Wright (1978) allowing selective growth of fusion products by complementation of artifically induced metabolic blocks. Single mammalian hybrid cells are capable of growth in the absence of other viable cells. In an attempt to determine if the same procedure could be used to isolate higher plant somatic hybrids Nehls (1978) treated protoplasts of Solanum nigrum and Petunia hybrida with two different biochemical inhibitors, diethylpyrocarbonate or iodoacetate. Two cells from fusion experiments were observed to divide 2 to 3 times, but these subsequentially budded and died. Whether this result was due to a deficiency in the system when it is applied to higher plants or a possible cellular incompatibility between the parents used, might be determined by using parental protoplasts known to be compatible in somatic hybridization.

CONCLUSIONS

To test the hypothesis that it is possible to select double-resistant somatic cell hybrids after fusing protoplasts of different amino acid analogue-resistant cell lines, Nicotiana sylvestris cell lines resistant to S-2-aminoethyl-cysteine (AEC^R), or 5-methyl-tryptophan ($5MT^R$) were isolated in suspension culture. Single spontaneous resistant variants arose from 6×10^6 cells (AEC^R), and 10^7 cells ($5MT^R$), respectively, a frequency similar to that observed by other investigators. The resistant lines isolated in this study required in excess of 100 ($5MT^R$), or 1,000 (AEC^R) times as much of their respective analogues as the parent wild-type culture for total growth inhibition. The AEC^R line also exhibited resistance to 10 mM lysine plus threonine, which was totally inhibitory to growth of the $5MT^R$ line. From analysis of free amino acid levels of suspension cultures of the wild-type and resistant cell lines, it was concluded that examination of only the "target" amino acid could lead to false results. The AEC^R line showed not only an increase in lysine, but also similar increases in other amino acids, some not aspartate-derived. Examination of free lysine levels in protoplast-derived calli indicated that the AEC^R line does not accumulate free lysine. A study of the aspartate pathway feedback control enzymes to determine the mechanism of this AEC-resistance is needed. The $5MT^R$ cell line is an apparent overproducer of free tryptophan, but a study of anthranilate synthetase is required to determine if this is due to relaxed feedback control.

Eight calli (0.8%) were selected on double analogue medium after heterokaryotic fusion of AEC^R and $5MT^R$ protoplasts. A total of 1.8 x 10^4 control calli from mixed AEC^R and $5MT^R$ protoplasts, and AEC^R and $5MT^R$ homokaryotic fusions, were placed on the double analogue selection, but none survived. It was concluded that there was no evidence for cross-feeding, high frequency appearance of double resistance within the parental cell lines, nor increased resistance with increased ploidy. Double resistant AEC^R + $5MT^R$ calli appeared only after fusion of parental protoplasts, and it was concluded that these were somatic cell hybrids.

Analysis of the hybrid calli showed the AEC-resistance to be dominant, and the 5MT-resistance semidominant. The hybrid cells exhibited levels of free tryptophan intermediate between that of the parental cell types. AEC^R and $5MT^R$ suspension cells had a distinct and nonoverlapping difference in chromosome numbers, and the hybrid calli examined had chromosome numbers higher than either parent. The expression of an AEC^R cell line trait, the ability to synthesize chlorophyll in the normally inhibitory presence of 2,4-D, was intermediate in quantity, but the same qualitatively, in the hybrid H2. None of the protoplast-derived calli examined were auxin-independent in their growth.

To the best of my knowledge this is the first report of the use of amino acid analogue resistant cell lines to select somatic cell hybrids in plants or animals. A partial report of this work is currently in press (White and Vasil 1979).

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Professor of Microbiology

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